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THE REPRESSION OF MEF2 TRANSCRIPTION FACTORS EXERTED BY CLASS IIA HDACS AND THEIR DEGRADATION STIMULATED BY CDK4 DETERMINE THE ACQUISITION OF HALLMARKS OF TRANSFORMATION IN FIBROBLASTS.

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Eros Di Giorgio

To my family, Sara and those who believe in the research against cancer

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ABSTRACT

The repression of MEF2 transcription factors exerted by class IIa HDACs and their degradation stimulated by CDK4 determine the acquisition of hallmarks of transformation in fibroblasts.

MEF2 transcription factors (TFs) are well known regulators of differenziative and adaptive responses, with predominant roles in muscular, cerebral and immune districts. However, literature concerning the contribution of MEF2 TFs in processes of transformation and oncogenesis is scattered and contradictory; class IIa HDACs (HDAC4, HDAC5, HDAC7, HDAC9) are well-established repressors of MEF2 activity and increasing numbers of selective class IIa HDACs inhibitors are under preclinical screening for various diseases, including cancer. However, a clear demonstration of the oncogenic functions of these proteins is still missing. The aim of this work was to clarify the possible involvement of the HDAC-MEF2 axis in carcinogenesis using as a model different mesenchymal cell lines with varying degrees of immortalization. Here, we incontrovertibly demonstrate a pro-oncogenic role of a nuclear resident form of HDAC4/HDAC7 in NIH-3T3 and BALB/c fibroblasts. Through a DNA microarray experiment we identified the signature of HDAC4 and, as expected, among the genes directly repressed by HDAC4 many are MEF2 targets. We demonstrated that most of the transforming potential of HDAC4 is due to the repression of MEF2 transcriptional activity and that the MEF2-HDAC axis is particularly active in Soft-tissue Sarcomas; in these tumors the binding between HDAC4 and MEF2 could be an effective therapeutic target, as proved by us *in vitro.*

We also demonstrated that the repression of MEF2 activity could also be exerted by common oncogenes, such as RAS and AKT, which act independently from class IIa HDACs by inducing a decrease in the half-life of MEF2C and MEF2D proteins. We reported that MEF2C/D are subjected to a cyclic degradation during cell-cycle with peaks of dysregulation concomitant with S phase entry. The signal that controls the cyclic degradation of MEF2 is the phosphorylation by CDK4/CyclinD1 on two serine residues, conserved among the MEF2 family members, except for MEF2B and a transcriptional variant expressed in skeletal muscles. As a consequence of this phosphorylation, MEF2C/D are bound by the E3-ligase SKP2 that mediates their poly-ubiquitylation and degradation in the proteasome. The cyclic degradation of MEF2 proteins is required for the correct progression of the cell-cycle, as any interference in this degradation process causes an arrest in G1 because of MEF2-mediated transcription of p21/CDKN1A; on the contrary, any increase in MEF2 degradation causes an aberrant progression in the cell-cycle, a common feature of cancer cells.

In summary, we demonstrated that in fibroblasts MEF2 activity could be alternatively repressed by class IIa HDACs or through a cell-cycle based degradation process; in both the cases MEF2 repression results in an increase in cell proliferation and in the acquisition of hallmarks of transformation.

RIASSUNTO

La repressione dei fattori di trascrizione MEF2 esercitata dalle Iston Deacetilasi di classe IIa e la loro degradazione stimolata da CDK4 determina l'acquisizione di caratteristiche maligne nei fibroblasti.

I fattori di trascrizione MEF2 sono regolatori ben noti di risposte differenziative e di adattamento, con ruoli predominanti nei distretti muscolari, cerebrali e del sistema immunitario. Tuttavia, la letteratura riguardante il contributo dei fattori MEF2 nei processi di trasformazione e di oncogenesi è contraddittoria; le Iston Deacetilasi di classe IIa (HDAC4, HDAC5, HDAC7, HDAC9) sono forti repressori dell' attività dei fattori MEF2 e un numero crescente di inibitori selettivi di queste proteine stanno entrando in fase clinica per l'applicazione sperimentale in diverse patologie, compreso il cancro. Tuttavia, una chiara dimostrazione delle funzioni oncogeniche di queste proteine è ancora mancante. Lo scopo di questo lavoro è stato quello di chiarire il possibile coinvolgimento dell'asse HDAC4/7-MEF2 nella carcinogenesi utilizzando come modello diverse linee di cellule mesenchimali a vari gradi di immortalizzazione. Qui, dimostriamo incontrovertibilmente un ruolo pro-oncogenico di una forma residente nucleare di HDAC4 e HDAC7 in fibroblasti murini NIH-3T3 e BALB/c. Mediante un esperimento di DNA microarray abbiamo identificato la firma genica di HDAC4 e, come previsto, tra i geni repressi direttamente da HDAC4 molti sono regolati da MEF2. Abbiamo dimostrato che la maggior parte del potenziale trasformante di HDAC4 è dovuta alla repressione dell'attività trascrizionale di MEF2 e che l'asse HDAC4/7-MEF2 è particolarmente attivo nei sarcomi dei tessuti molli; in questi tumori il legame tra HDAC4 e MEF2 potrebbe essere un bersaglio terapeutico efficace, come dimostrato da noi in vitro.

Abbiamo anche dimostrato che oncogeni comuni, quali RAS e AKT, reprimono l'attività di MEF2 agendo in un modo indipendente dalle HDAC di classe IIa e destabilizzando a livello proteico MEF2C e MEF2D. In particolare, MEF2C/D sono sottoposti ad una degradazione ciclica ad ogni ciclo cellulare con punte di disregolazione in concomitanza all'ingresso in fase S. Il segnale che controlla la degradazione ciclica dei MEF2 è la fosforilazione da parte di CDK4/CyclinD1 su due residui conservati tra i membri della famiglia MEF2, tranne che da MEF2B e da una variante trascrizionale diffusa nei muscoli scheletrici. Come conseguenza di questa fosforilazione, MEF2C/D vengono legati dall'E3 ligasi SKP2 che media la loro poli-ubiquitinazione e degradazione nel proteasoma. La degradazione ciclica delle proteine MEF2 è necessaria per la corretta progressione del ciclo cellulare e qualsiasi interferenza in questo processo di degradazione altamente regolato provoca un arresto in G1 a causa dell'induzione da parte di MEF2 del regolatore negativo del ciclo p21/CDKN1A; al contrario, un aumento della degradazione di MEF2 provoca una progressione aberrante nel ciclo cellulare, una caratteristica comune delle cellule tumorali.

In sintesi, abbiamo dimostrato che nei fibroblasti l'attività trascrizionale dei fattori MEF2 può essere alternativamente repressa dalle HDAC di classe IIa o mediante un processo di degradazione scandita dal ciclo cellulare; in entrambi i casi la repressione dei fattori di trascrizione MEF2 determina un aumento della proliferazione cellulare e l'acquisizione di caratteristiche maligne anche da parte di cellule sane.

INTRODUCTION

1. The HDACs world

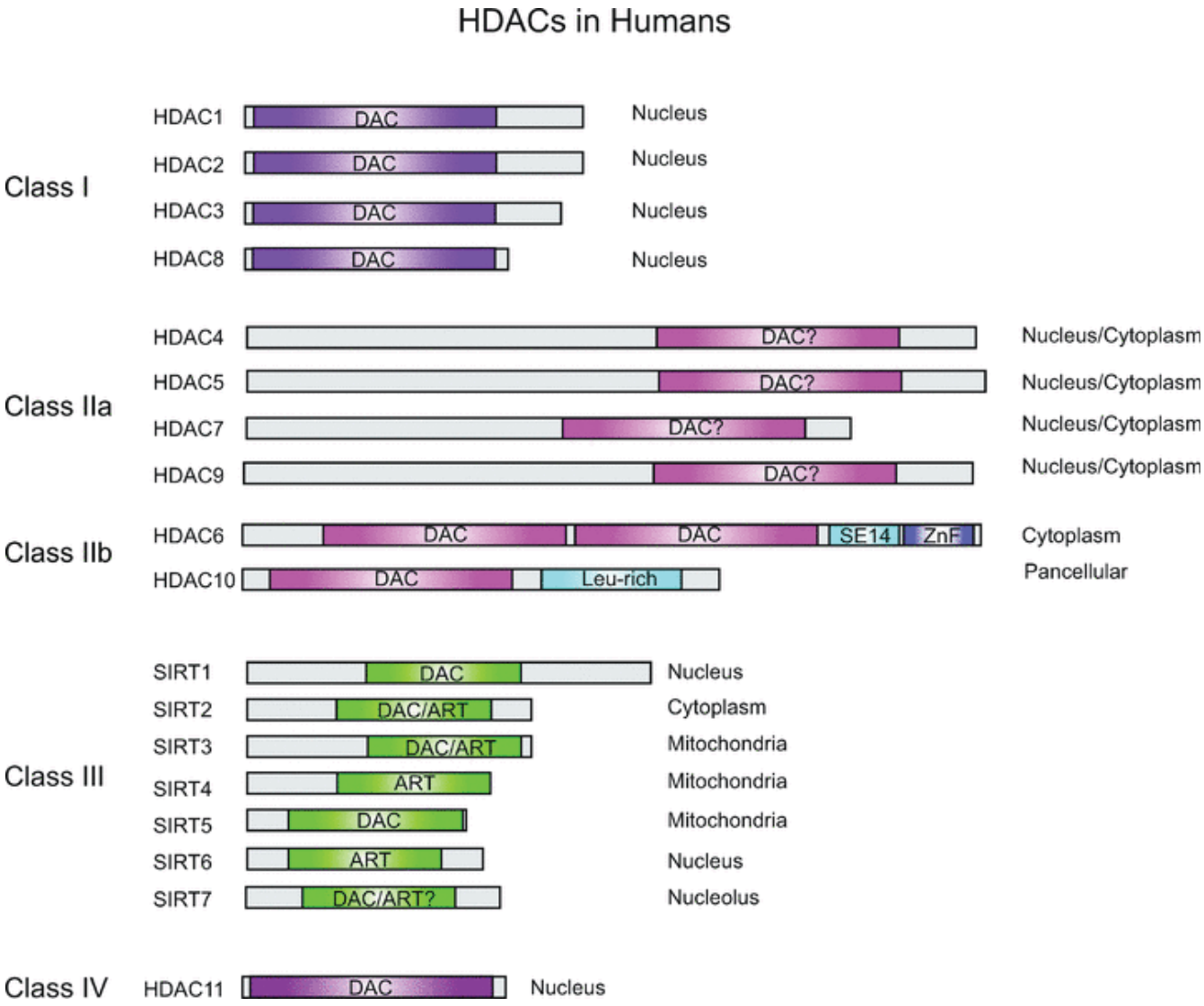

Figure 1 The HDACs world: Representation of the main features of the 18 mammals HDACs and indication of their sub-cellular localization (from Clocchiatti et al, 2011).

To date in mammals 18 Histone DeACetylase (HDACs) have been identified, divided into four families on the basis of their sequence homology and phylogenetic criteria (Figure 1). The four classes also differ profoundly for cellular localization and enzymatic activity (reviewed by Martin et al, 2007).

The class I include HDAC1, 2, 3 and 8, which are homologous to the yeast Rpd3; they are Zn^{2+}-dependent, ubiquitously expressed and their sub-cellular localization is the nucleus (Martin et al, 2007).

The class II comprises HDAC4, 5, 6, 7, 9 and 10, which are homologous to the yeast HdaI; they are Zn^{2+}-dependent, mainly expressed in muscle tissue, bone, neurons, in the endothelium and in thymocytes. On the

basis of structural and functional characteristics, the class II has been further divided into sub-class IIa (HDAC4, 5, 7, 9), which includes deacetylases enzymatically inactive and characterized by nuclear cytoplasmic shuttling, and IIb (HDAC6 and 10), that are instead enzymatically active on some substrates such as tubulin (Martin et al, 2007).

Class III HDACs or Sirtuins (SIRT1, 2, 3, 4, 5, 6, 7) are very different from the previous ones, as they are NAD + - dependent enzymes. They are homologous to the yeast Sir2 and they are pan-localized inside the cells in nuclear, nucleolar, cytoplasmic and mitochondrial compartments (Bosch-Presegué and Vaquero, 2014). In the last few years this class of proteins has become extremely studied because of important roles in maintaining the genome stability, in metabolisms and aging (Bosch-Presegué and Vaquero, 2014).

HDAC11, having characteristics intermediate between class I and II, is considered separately as a deacetylase of class IV (Martin et al, 2007).

2. Class IIa HDACs: similarities and differences between class IIa and class I HDACs

Class I and class IIa HDACs share most of the catalytic domain at the carboxy-terminus. However, class IIa HDACs are characterized by an extended amino-terminal region, absent in class I HDACs (Zhang et al, 2008). This class IIa N-terminus region is quite conserved among the four members, with a homology of 30-40%, except for some few key amino acid residues (Martin et al, 2007; Di Giorgio et al, 2015). Taking HDAC4 as a representative member of class IIa HDACs, I'll describe below the typical organization of class IIa proteins starting from the N-terminus and moving towards the C-terminus (Figure 2).

At the N-terminus there is the nuclear localization signal *nls2* (amino acids 1-117 in HDAC4), followed by the binding site for the corepressor CtBP (C-terminal binding protein, amino acids 48-52 in HDAC4), absent in HDAC7 (Fischle et al, 2001), and a coiled coil region rich in glutamine (aa 62-129 in HDAC4, also absent in HDAC7), which is the binding site for many transcription factors including MEF2 and RUNX2, and which precedes the second nuclear localization signal *nls1* (aa. 244-279) (Han et al, 2005, Guo et al, 2007, Nishino et al, 2008). HDAC4, 5 and 9 can interact by means of a region of a hundred amino acids, comprised between amino acids 400 and 500, with HP-1α (Heterochromatin Protein 1), an adapter protein that recognizes the methylation signal on lysine 9 of histone H3 and recruits the methyltransferase SUV39H1, thus propagating and perpetuating the methylation and the heterochromatinization (Zhang et al, 2002 A). CtBP seems to collaborate in the recruitment of the methyltransferase and of other enzymes and co-repressors (Dressel et al 2001). In particular it has been demonstrated that HDAC4, 5 and MITR, a splice-variant of HDAC9, interact both *in vitro* and *in vivo* with CtBP (Zhang et al, 2001, Dressel et al, 2001). CtBP recruits also class I HDACs (HDAC1, 2 and 3), increasing the effectiveness of the repressive action of class IIa HDACs on MEF2 (Zhang et al, 2001).

The carboxy-terminal domain contains the HDAC-defective domain (aa 665-1084 in HDAC4), the binding site for the complex N-CoR/SMRT-HDAC3 (Fischle et al, 2002) and the *nes (*nuclear export signal), which mediates the nuclear export via CRM-1 (McKinsey et al, 2001).

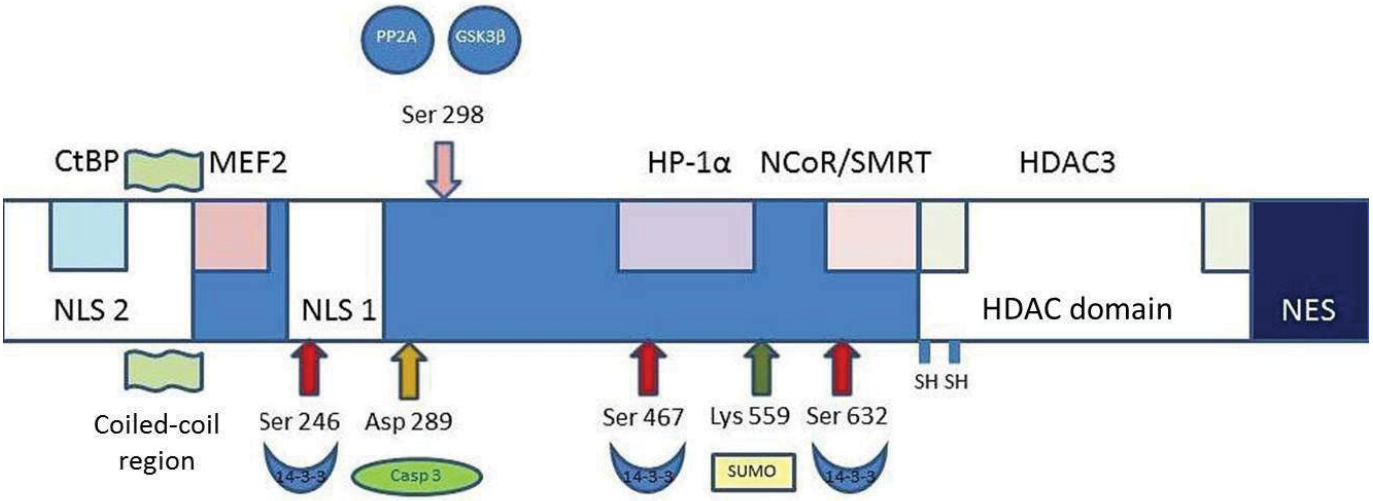

Figure 2 Schematic representation of HDAC4 domains. Further details are explained in the text.

Class I and class IIa HDACs differs at molecular and functional levels for big differences in two domains:

a) the N-terminus domain, that is absent in class I HDACs, mediates the interaction with specific partners. Since these proteins are not capable of direct DNA binding, any association between a nucleic acid and a class IIa HDAC should be mediated by a third partner (Wang et al, 1999). Moreover, in the N-terminus, as discussed in details in the following paragraphs, lies the 14-3-3 proteins binding domain, that mediate the nucleus-cytoplasmic shuttling of the deacetylases, which is one of the best characterized strategy of regulation of their action (Martin et al, 2007).

b) the C-terminus HDAC domain, that is defective and enzymatically "inactive" (or at least not active on classical substrates). The overall structure of class IIa HDAC domain is quite similar to class I; in fact the predictive structure of class IIa HDAC domain made on the assumption of similarity between class I and IIa catalytic core, the so-called Finnin-model (Finnin et al, 1999), that was proposed more than 15 years ago, is in general correct. Class IIa HDAC domain is made up of approximately 400 residues (aa) arranged into 21 α-helices and 10 βsheets organized in a single domain, structured around a central ''catalytic'' Zn^{2+} ion (Schuetz et al, 2008). Similarly to class I HDACs, 2 aspartates and an histidine coordinate this Zn^{2+}, while 2 other aspartates, an histidine, a serine and a leucine coordinate two potassium ions (Vannini et al, 2004; Schuetz et al, 2008; Bottomley et al, 2008). Despite this high similarity, class IIa HDACs possess a bigger active site than class I HDACs. This is due to an aminoacid substitution in the catalytic site (Y976H in HDAC4) of class IIa HDACs that makes these proteins enzymatically inactive on classic substrates such as the acetylated lysines of histone tails (Lahm et al, 2007; Figure 2). This change is evolutionary conserved among class IIa HDACs and typical of vertebrates and its degree of conservation is actually an evolutionary mystery (Gregoretti et al, 2004; Lahm et al, 2007). From a chemical point of view, the substitution of a tyrosine typical of class I HDACs with an histidine that is sterically less cumbersome causes the relaxation of the Zn binding domain during the classical reaction of deacetylation. The Zn^{2+} is therefore too far from the histidine which, in respect to class I tyrosine, becomes unable to form hydrogen bonds with the intermediate of the enzymatic reaction. As a matter of fact the intermediate is not properly stabilized and the reaction results ineffective (Lahm et al, 2007). Nevertheless, class IIa can efficiently process some not-natural halogenate compounds such as trifluoroacetyl-lysine. In this

case the presence of the trifluoro group stabilizes the amide bond, hence favoring the reaction even in the absence of transition-state stabilization (Lahm et al, 2007). Importantly, a gain of function mutant of HDAC4 in which histidine 976 is mutated into tyrosine, has a catalytic efficiency 1,000-fold higher compared to the wild-type form (Lahm et al, 2007; Bottomley et al, 2008). Nonetheless, this gain of function mutant does not show enhanced repression respect to the wild-type, at least in the instance of MEF2-dependent transcription, a well-known class IIa partner (Lahm et al, 2007). Being MEF2 transcription factors (TFs) the favorite class IIa HDACs partners, a substantial section of this thesis will be devoted to MEF2s.

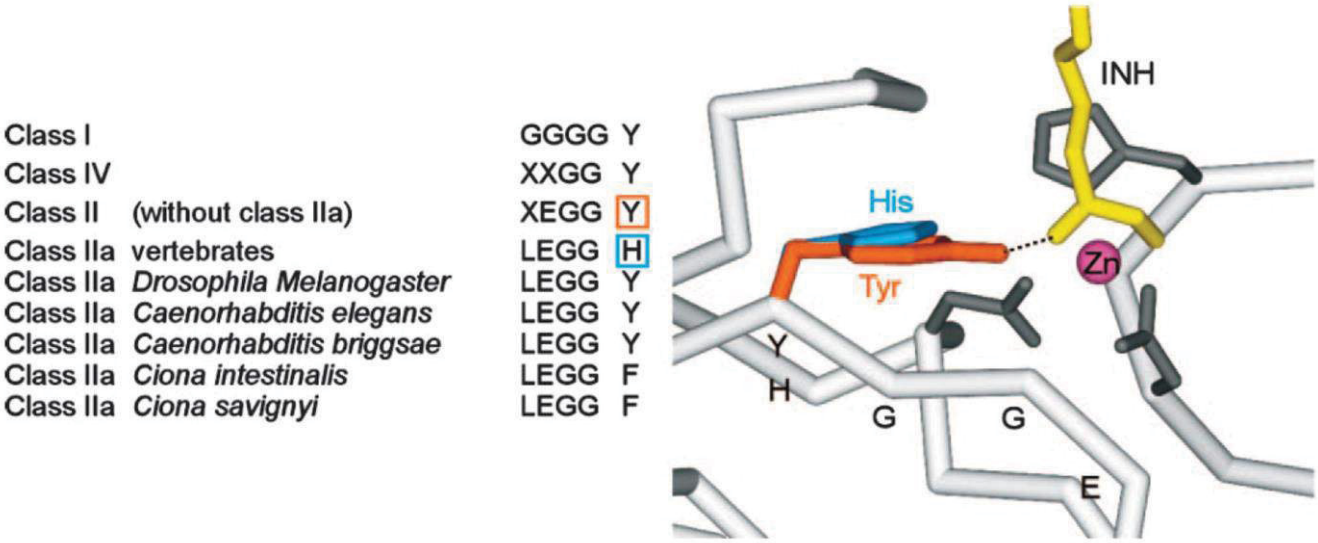

Figure 3 A point mutation in the catalytic site abolishes the HDAC activity of Vertebrates class IIa HDACs. Left: alignment of the aminoacids surrounding Histidine 976 of HDAC4. Right: the replacement of the tyrosine with an histidine in Vertebrates class IIa HDACs prevents the stabilization of the transition state during HDAC reaction. In the picture INH is a representative hydroxamic acid moiety (Adapted from Lahm et al, 2007).

Another distinctive feature of class IIa HDAC catalytic site is the existence of a Zinc Binding Domain (ZBD) which accommodates a second structural Zn^{2+}. This ZBD has a pocket-like shape formed by a β-hairpin surrounded by two antiparallel β-strands (Schuetz et al, 2008; Bottomley et al, 2008; Figure 4). The structural Zn2+ atom in the center of the pocket is coordinated by three cysteines (667, 669, 751 in HDAC4) and one histidine (675 in HDAC4) (Bottomley et al, 2008). This domain is extremely flexible and the oxidation of the cysteines involved in Zn^{2+} coordination (667 and 669 in HDAC4) is sufficient to free the metal and open and deconstruct the ZBD (Bottomley et al, 2008). As a matter of fact class IIa HDACs are extremely sensitive to redox conditions and it is reported that in HDAC4 the oxidation of cysteines 667 and 669 induces the formation of a disulphide bond that causes the de-structuration of the ZBD, the exposition of the *nes* with the subsequent export in the cytoplasm and the detachment of HDAC3 (Ago et al, 2008). During cardiac hypertrophy there is an increase of ROS production, which causes the nuclear export of HDAC4 (Ago et al, 2008). The two cysteines of HDAC4 could be targeted with anti-oxidant drugs in order to promote the nuclear re-localization of HDAC4 and the inhibition of pro-hypertrophic stimuli (Ago et al, 2008). Recently, it has been demonstrated that Tasquinimod, a drug already known for its efficiency against prostate cancer, targets the ZBD of HDAC4 and mimics therefore the effects of oxidative stress, causing the de-structuration of the C-terminus of HDAC4 and the dissociation of HDAC3 (Isaacs et al, 2013).

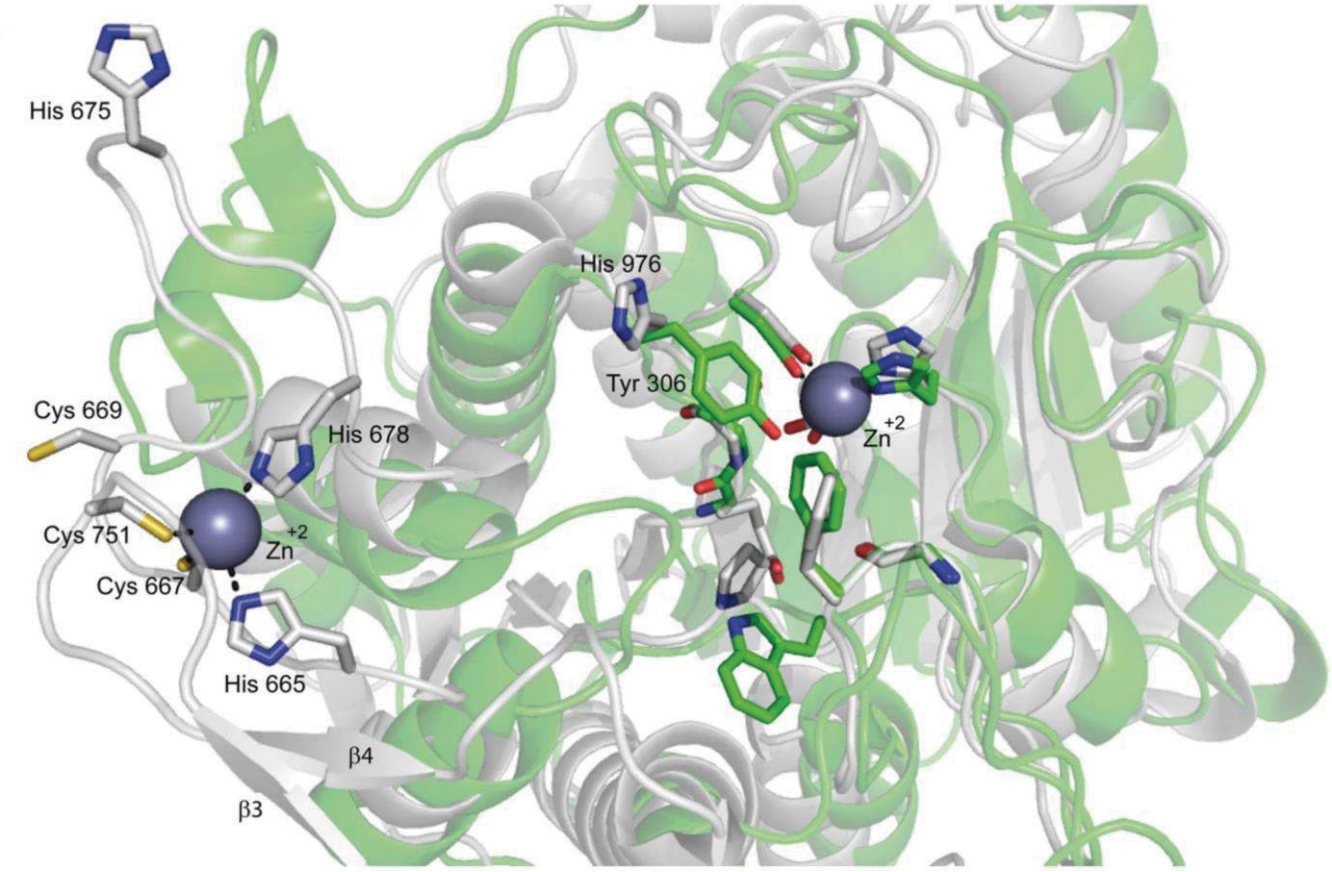

Figure 4 Superimposition of the ribbon structure of a class I HDAC (HDAC8, in green) and of the inhibitor (TFMK)-bound HDAC4 (in white). The picture shows the different orientation in respect to the Zn^{2+} of HDAC8 Tyr 306 and of HDAC4 His976. On the left it is shown the second Zn^{2+} ion, the so called structural Zn^{2+}, typical of Vertebrates class IIa HDACs (from Di Giorgio et al, 2015).

3. Class IIa HDACs: HDACs with orphan substrates or missed during evolution?

In between 2002 and 2008, at IRBM (Pomezia, Rome), a Merck-research institute, scientists were involved in the study of the catalytic site of class IIa HDACs. They provided wonderful demonstrations of the ineptitude of class IIa HDACs in deacetylating classical substrates such as the histones (Fischle et al, 2002; Lahm et al, 2007; Bottomley et al, 2008). In particular they demonstrated that any enzymatic activity associated to class IIa HDACs should be explained by the recruitment of class I enzymes and in particular of the HDAC3-SMRT/N-CoR complex (Fischle et al, 2002). In fact, the study of class IIa enzymatic activity starting from the proteins purified from mammalian cells is extremely difficult because of frequent contamination of class I HDACs (Fischle et al, 2002; Clocchiatti et al, 2013 A and B). All HDAC4 point mutants that abrogate its apparent enzymatic activity (H803A, G811A, D838A, D840A, H842A, N845D, D934 N, E973G) are due to a lack in the recruitment of HDAC3 (Fischle et al, 2002). Moreover, a classic deacetylase activity is not associated with the cytoplasmic fractions of either HDAC7 or HDAC4 as these forms are only weakly associated to the mainly nuclear HDAC3 (Fischle et al, 2001). A still open question regards how much of the HDAC3-associated activity is necessary to class IIa HDACs in order to carry out their functions. In fact it was demonstrated that the fraction of active HDAC3 that interacts with HDAC4 is extremely low (Yang et al, 2002; Han et al, 2005).

For certain activities the HDAC domain is completely dispensable, for example for the repression of RUNX2 and SRF and the amino-terminus nuclear deletion (ΔC) generated by caspase cleavage is a stronger repressor than the wild-type (Paroni et al, 2007). Instead, for the repression of MEF2, the C-terminus is apparently required as the ΔC results less repressive than the wild-type and much less in respect to another nuclear resident form mutated in the 14-3-3 binding sites (TM) (Paroni et al, 2007); however the GOF mutant of HDAC4 in which the histidine 976 is replaced by a tyrosine is as repressive as the wild-type (Lahm et al, 2007). The explanation to this apparent contradiction is that the repression of MEF2 activity requires a strong retention on the chromatin and the ΔC in respect to the TM is much less bound to chromatin (Paroni et al, 2007); secondly, MEF2 repression could require the HDAC activity of HDAC3 that interacts with HDAC4 through the C-terminus (Fischle et al, 2002). The generation of a TMΔC mutant should discriminate which of these two effects is the stronger.

As reported by Yang group, class IIa HDACs possess at least two repressive domains, once located in the N-terminus and the other in the C-terminus (Wang et al, 1999). TSA treatment decreases only the repressive effect of the C-terminus but not of the N-terminus, and the effect of the N-terminus is much stronger compared to the C-terminus, thus confirming the great importance of the N-terminus in the repression of some TFs such as MEF2 (Wang et al, 1999).

However, as anticipated, class IIa HDACs are not completely inactive since, in virtue of their mutated catalytic site, they are able to remove the acetyl moiety from halogenate compounds, such as trifluoroacetyl-lysine (Bottomley et al, 2008).

An open question is if in nature a compound analogous to trifluoroacetyl-lysine does exist.

If all the HDAC activity attributed to class IIa HDACs inside the nucleus could be related to class I HDACs and in particular to HDAC3, there are at least four cytoplasmic partners that are reported to be deacetylated by HDAC4 in the cytoplasm: HIF-1α, STAT-1, MEKK2 (Clocchiatti et al, 2013 A) and DNAJB6/8 complex (Hageman et al, 2010). The origin of this catalytic activity of HDAC4 in the cytoplasm is still mysterious.

HIF-1α (Hypoxia Inducible Factor 1α) is a DNA binding transcription factor highly degraded under normoxic conditions by the E3-ligase VHL that operates under the oxygen-dependent prolyl hydroxylation. Hif-1α is instead stabilized under hypoxia (Jeong et al, 2002).

Two major sites of Hif-1α have been recognized as acetylated: Lys-532 and Lys-674 (Jeong et al, 2002; Lim et al, 2010). The acetylation at Lys-532 is inhibitory because it leads to VHL-dependent HIF1α ubiquitylation and degradation (Jeong et al, 2002). In contrast, the acetylation at Lys-674 is activating and it is required for HIF-1 transcriptional activity (Lim et al, 2010). Class IIa HDACs contribute to activate Hif-1α by acting on both these lysines:

a) the cytoplasmic fraction of class IIa HDACs increase the stability of Hif-1α by removing the acetyl-moieties affixed to HIF-1α on lysine 532 by the acetyl-transferase ARD1 (Chen and Cepko, 2009) and by removing other acetyl-moieties on lysines 10, 11, 12, 19, 21 (Geng et al, 2011). In particular Chen and Cepko demonstrated in 2009 that HDAC4 has a pro-survival role during normal retinal development in bipolar (BP) interneurons (Chen and Cepko, 2009). This property is common only to the cytoplasmic fraction of HDAC4 not bound to MEF2 and it is due to the HDAC4-dependent deacetylation and stabilization of Hif-1α (Chen and Cepko, 2009). A reduction in HDAC4 expression during normal retinal development led to apoptosis of rod photoreceptors and bipolar (BP) interneurons, whereas overexpression reduced naturally occurring cell death of the BP cells. Geng and colleagues, working on hepatocarcinoma and prostate cancer cell lines, described a similar action of HDAC4, but in these circumstances on residues in the N-terminus of Hif-1α (Geng et al, 2011). Hif-1α

stabilization has oncogenic effects, as it promotes the transcription of VEGF and sustains glycolysis (Geng et al, 2011).
b) the nuclear fraction of class IIa HDACs increases the transcriptional activity of Hif-1α by stimulating the acetylation by p300 of lysine 674 (Kato et al, 2004; Seo et al, 2009).
Class IIa HDACs are also able to interact with Hif-1α on the binding site for FIH-1, an inhibitor factor of Hif-1α transcriptional activity, thus displacing FIH-1 (Seo et al, 2009), promoting the interaction with p300 (Kato et al, 2004) and stimulating the acetylation of lysine 674 (Seo et al, 2009). This mechanism, which seems to be active mainly during hypoxia, has no relevant impact on Hif-1α degradation and requires the nuclear re-localization of the complex formed by class IIa HDACs and Hif-1α (Kato et al, 2004).
Curiously, MEF2C has been identified as a transcriptional target of HIF-1α in cardiomyocytes taken from developing hearth (Krishnan et al, 2008) and this could suggest a buffering role of MEF2-HDAC axis in the regulation of Hif-1α and cardiomyogenesis (Clocchiatti et al, 2013 A).

Similarly to Hif-1α, also STAT-1 has been reported to be deacetylated by HDAC4 in the cytoplasm of ovarian cancer cells resistant to cisplatin (Stronach et al, 2011). Curiously, this deacetylation takes place only in the cytoplasm of resistant cells and the activation of STAT-1 contributes to the resistance of these cells to apoptotic cell death after cisplatin treatment (Stronach et al, 2011). These data were recently confirmed by a different research group (Kaewpiboon et al, 2014). They demonstrated that HDAC4 levels rise in A549 human lung cancer cells resistant to etoposide; this increase in HDAC4 causes the phosphorylation and activation of STAT-1 and the subsequent transcription of the multi-drug resistance related gene P-glycoprotein (Kaewipiboon et al, 2014). Overall, it seems that HDAC4 could mediate resistance to chemotherapy mainly through the modulation of the JAK-STAT pathway. Considered the potential impact of these findings in oncology, further investigations are required to confirm the centrality of STAT-1 in this phenomenon and to prove the absolute contribution of HDAC4 deacetylase activity in the regulation of STAT-1. It has been reported that HDAC4 is implicated also in the deacetylation and activation of STAT-6 and the subsequent STAT-6 dependent transcription of *Arginase-1* in differentiating monocytes, thus promoting their full differentiation (Yang et al, 2014).

During muscle denervation, HDAC4 activates an atrophic program by deacetylating the MAPK MEKK2 on lysine 385, thus promoting its phosphorylation and activation (Choi et al, 2012). In particular, during denervation HDAC4 accumulates and activates MEKK2 (Choi et al, 2012). The activation of MEKK2 culminates in the activation of AP-1 and the subsequent production of cytokines and muscle remodeling (Choi et al, 2012). As for Hif-1α and STAT-1, also MEKK2 deacetylation requires the cytoplasmic localization of HDAC4, which exclude a putative contribution of HDAC3 in the reaction of deacetylation (Choi et al, 2012). Intriguingly, STAT-1 and MEKK2 deacetylation seems to be a property restricted only to HDAC4, among the class IIa HDACs (Stronach et al, 2011; Choi et al, 2012).

Recently it was demonstrated that in the presence of genotoxic stress, at early times HDAC5 is involved in the deacetylation of K120 of p53 (Sen et al, 2013); this event blocks p53 on the promoters of anti-proliferative genes. Prolonged DNA damage (24h of treatment with etoposide) causes the CaMK-mediated export of HDAC5 and allows the acetylation of K120 of p53, which is recruited on the promoters of pro-apoptotic genes and induces apoptosis (Sen et al, 2013); the authors therefore proposed the regulated nuclear export of HDAC5 as a switcher of p53 functions from anti-proliferative to pro-apoptotic. These findings are surprising not only for the reported capability of HDAC5 to catalyze the deacetylation of a nuclear protein, but also for the attribution to a class IIa HDAC of a strong pro-survival anti-proliferative effect (Sen et al, 2013).

Finally, it is reported that HDAC4 is required for the full anti-aggregation activity of DNAJB6b and DNAJB8. In particular HDAC4 stimulates the processing of DNAJB6/8 substrates by directly deacetylating the lysines K216 and K223 of DNAJB6 and 8 and thus increasing their activity (Hageman et al, 2010).

Another peculiar aspect is that for the interaction of a class IIa HDAC with these four partners the N-terminus seems to be dispensable (Kato et al, 2004; Stronach et al, 2011; Choi et al, 2012).

4. Pathways of regulation

Class IIa HDACs activity is regulated both at a transcriptional and at post-transcriptional levels. The capability of these proteins of nucleus-cytoplasmic shuttling offers to class IIa HDACs an additional level of regulation in respect to class I HDACs. Finally, the enzymatic deficiency of class IIa HDACs makes them dependent on class I HDAC associated activity for certain functions, offering a third level of regulation.

a) regulation of class IIa HDACs transcription and modulation of the stability of the messengers (RNAi)

Transcriptionally, the expression of HDAC4 is repressed by mithramycin, a well-known antibiotic able of binding DNA sequences at GC repetition in the presence of Mg^{2+} ions (Sleiman et al, 2011). Mithramycin therefore acts on the promoter of HDAC4, rich in GC, and displaces the transcription factors Sp (Liu et al, 2006). The interaction between the promoter of HDAC4 and Sp1 and Sp3 has been confirmed by EMSA and ChIP. Moreover, by increasing the expression of Sp1 and Sp3, the expression of HDAC4 increases. Conversely, the silencing of Sp1 and Sp3 causes the concomitant repression of HDAC4 (Liu et al, 2006).

Despite this general Sp1/Sp3 dependent regulation of class IIa HDACs transcription, several other circuits are involved in regulating their transcription.

I'll further describe in details the metabolic shift induced by HDAC4 in denervated muscles (Tang et al, 2009). In this paper Tang and colleagues demonstrated that HDAC4 is induced in atrophic muscles (Tang et al, 2009). This induction is mainly due to a feed-forward mechanism. The initial nuclear re-localization of HDAC4 in atrophic muscles causes the activation of Myogenin. Myogenin in turn induces HDAC4 transcription alimenting this feed-forward mechanism (Tang et al, 2009).

A similar loop has been described by Olson group during muscle differentiation (Haberland et al, 2007). In this case MEF2A, MEF2C and MEF2D are able to bind the promoter of *HDAC9* and induce its expression, thus buffering the rate of differentiation (Haberland et al, 2009). It is not clear at the moment if MEF2 regulates directly also the transcription of other class IIa HDACs.

It is possible that these strategies could be extremely useful when regulation of the sub-cellular localization of class IIa HDACs is not sufficient to achieve a strong effect.

This is the case of stemness regulation. It is reported that in embryonic stem cells, class IIa HDACs behave as negative regulators of stemness, possibly thought he repression of *Oct3/4* and *Klf4* genes (Addis et al, 2010). Oct3/4 in embryonic stem cells prevents the HDAC4-mediated repression by binding the first intron of HDAC4 and interfering with the correct splicing (Addis et al, 2010).

The regulation of the stability of class IIa transcripts offers another tool to impact on these enzymes. As a matter of fact class IIa HDACs are targets of several miRNAs and several of them have been discovered by studying muscular and chondrocyte differentiation.

As previously described, during myogenesis MEF2 is able to induce the expression of *HDAC9* that acts by repressing its activity (Haberland et al, 2007). On the other hand, MEF2 also induces the expression of a miRNA,

miR-1, which targets *HDAC4* mRNA, fueling a positive feedback (Chen et al, 2006). miR-1 is required for the correct fusion of myoblasts, it is induced by the mTOR pathway and has pro-hypertrophic properties (Sun et al, 2010). miR-1 is also repressed in several cancers, such as hepatocellular carcinoma where its repression is associated to the up-regulation of FoxP1, MET, and HDAC4 (Datta et al, 2008) and lung cancer (Nasser et al, 2008). A similar up-regulation of HDAC4 was identified in several HCC samples characterized by reduced expression of miR-22 (Zhang et al, 2010). Overexpression of miR-22 is also sufficient to induce cardiomyocyte hypertrophy, especially through HDAC4 and Sirt1 down-modulation (Huang et al, 2013). miR-155 has oncogenic properties and it is particularly over-expressed in diffuse large B-cell lymphoma (Sandhu et al, 2012). As I will further analyze in details, this tumor is characterized by a strong up-regulation of MEF2 functions and *BCL-6* transcription (Ying et al, 2013).
HDAC7 is a target of miR-140-5p in tongue squamous cell carcinoma (Kai et al, 2014).
TGF-beta represses myogenesis and muscle differentiation. In C2C12 cells, TGF-beta inhibits muscle differentiation, through the repression of miR-206 and miR-29 and thereby by augmenting HDAC4 expression (Winbanks et al, 2011).
miR-1 stimulates also chondrocyte hypertrophy through HDAC4 targeting (Li et al, 2014). In a similarly manner acts miR-2861 that selectively target HDAC5 (Li et al, 2009). Another miRNA up-regulated during chondrocytes differentiation is miR-365. In particular, miR-365 is mechanosensitive and determines the induction of Hh and collagen X, the latter through direct targeting of HDAC4 (Guan et al, 2011).
miR-140 inhibits the proliferation and increases the chemo-resistance of p53 wild-type osteosarcoma cells, by inducing p53 and targeting HDAC4 (Song et al, 2009). MicroRNA-206 through HDAC4 repression delays amyotrophic lateral sclerosis progression and promotes regeneration of neuromuscular synapses (Williams et al, 2009).
Class II HDACs are also able to regulate the transcription of some miRNAs. In particular they promote the activation of hepatic stellate cells by promoting the transcription of miR-29 (Mannaerts et al, 2013). HDAC9 promotes angiogenesis at least in part by inhibiting the transcription of the anti-angiogenic miRNA miR-17-92 (Kaluza et al, 2013).
Finally, the mRNA of HDAC4 is down-regulated after aminoacid starvation (Palmisano et al, 2012). This HDAC4 down-regulation causes the derepression of silenced transgenes, including integrated plasmids and retroviruses (Palmisano et al, 2012); the causes of such dysregulation are not clear up to now.

b) sub-cellular localization

Class IIa HDACs show the peculiarity of shuttling between the nucleus and cytoplasm. Since they exert their function mainly in the nucleus (Fischle et al, 2002), their accumulation into the cytoplasm is general considered as a negative regulation, at least as transcriptional co-repressors for certain TFs (Martin et al, 2007; Clocchiatti et al, 2013). The nuclear localization of IIa HDACs prevails in undifferentiated cells, while the cytoplasmic one in differentiated cells, in muscle cells during hypertrophy, in hepatocytes during gluconeogenesis and in the granule cells (McKinsey et al, 2001; Backs and Olson, 2006; Mihaylova et al, 2011). Class IIa HDACs could also be localized into the mitochondria (HDAC7, Bakin et al, 2004), or in nuclear structures called "HDAC bodies associated to the matrix" (HDAC5, McKinsey et al, 2001) or in nuclear foci associated with DNA repair complexes and proteins such as 53BP1 (HDAC4, Kao et al, 2003), although these reports require confirmation.
HDAC4, like the other class IIa HDACs, has:

- two distinct *nls* (nuclear localization signal) placed in the N-terminus, with *nls1* that is rich in basic aminoacids and is located between aa 244 and 279, and *nls2*, that is less stronger and bigger (aa 1-117).
- a *nes* (nuclear export signal) in the C-terminus between aa 1054 and 1084 (McKinsey et al, 2001 and Nishino et al, 2008).

By varying the availability of the *nls* to bind to importin-α and of the *nes* to bind to CRM1, cells modulate HDAC4 protein localization. The rapid kinetics and the high reversibility of the phenomenon led scientists to speculate that the "shuttling" was mediated through phosphorylation, as this PTM is extremely quick and easily reversible by phosphatases. The goodness of this theory was confirmed in 2000 when multiple experimental evidences joined the nuclear export with the action of CaMKII (McKinsey et al, 2001). Later on the nuclear import was associated to the activity of the phosphatase PP1 and PP2A (Martin et al, 2007; Paroni et al, 2008; Kozhemyakina et al, 2009).

Nowadays, it is universally accepted that the model requires the phosphorylation of at least three (four in HDAC7) serines conserved among HDAC 4, 5, 7 and 9 (HDAC4: S 246, 467, 632; HDAC5: S 259,497,661; HDAC7: S 155, 181, 321, 446; HDAC9: S 220, 451, 611); these phosphorylation events facilitate the binding by dimers of 14-3-3 chaperones (Grozinger and Schreiber, 2000; Figure 5).

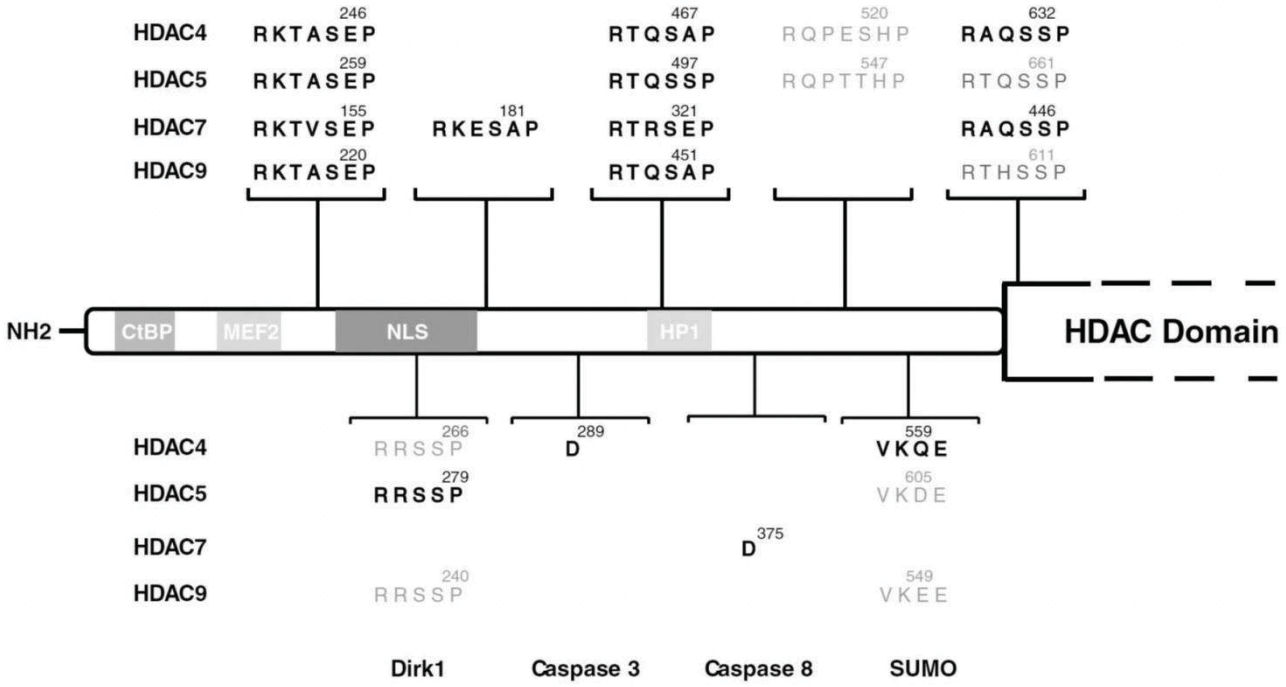

Figure 5 Class IIa HDACs are regulated through many signaling pathways. In figure, the 14-3-3 binding sites and the sites of caspases cleavage and of SUMOylation are highlighted. Motifs are written in bold when the corresponding post-translation modification was demonstrated experimentally (from Martin et al, 2007).

The binding to 14-3-3 proteins could either mask the *nls* preventing the nuclear import (Grozinger and Schreiber, 2000; Nishino et al, 2008), or unmask the *nes* and promote the direct interaction with CRM1 (Mc Kinsey et al, 2001), or both (although direct evidence of an interaction between HDAC4 and the CRM1 are not even available). It is still an open question where the interaction between 14-3-3 proteins and class IIa HDACs occurs; in the nucleus, in the cytoplasm or in both the compartments. The latter appears the most plausible hypothesis; in this case the nuclear 14-3-3 facilitate HDACs' export into the cytoplasm and cytoplasmic 14-3-3 prevent HDACs' nuclear re-localization (Nishino et al, 2008). Regardless of the molecular mechanism of the

"shuttling": i) the inhibition of the serine/threonine kinases implicated in the phosphorylation of the 14-3-3 sites, ii) the Ser to Ala mutation of these sites, or iii) the activation of phosphatases, all determine a nuclear accumulation of class IIa HDACs, thus confirming that the mechanism of HDACs' export is actually dependent both on phosphorylation and on 14-3-3 proteins (Grozinger and Schreiber, 2000; Wang et al, 2000; Nishino et al, 2008; Paroni et al, 2008).

Among the serine/threonine kinases involved in HDACs phosphorylation, the kinases responsive to calcium are the enzymes historically associated to HDACs nuclear export. CaMKI and IV phosphorylate all class IIa HDACs and show preference for residues 246 and 467 of HDAC4 (and the corresponding aa in other deacetylases), while CaMKII preferentially phosphorylates the serine 467 and 632 of HDAC4 (McKinsey et al, 2001 and Backs et al, 2006 A and B). CaMKII phosphorylates and exports directly only HDAC4 among class IIa HDACs, because only HDAC4 has a CaMKII specific docking site centered on Arg601 (Backs et al, 2006 A). However, since HDAC4 can form heterodimers with HDAC5 and 9, but not with HDAC7 (Fischle et al, 2001; Backs et al, 2008), the association between HDAC4 and HDAC5/9 makes such proteins responsive to CaMKII (Backs et al, 2008). In particular HDAC4 interacts strongly with HDAC5 through its amino-terminal region (aa 66-208), which includes the coiled-coil glutamine-rich region and the binding site for MEF2 (aa 166-184) (Backs et al, 2008). The MEF2 binding site is dispensable for the interaction between HDAC4 and HDAC5 and, as the interaction between the two proteins is direct, the presence of MEF2 in the complex is irrelevant (Backs et al, 2008). The presence of an intact glutamine-rich region is instead necessary for the interaction and the lack of this region in HDAC7 (Guo et al, 2007) explains its inability to interact with HDAC4 (Fischle et al, 2001; Backs et al, 2008). In this manner HDAC4 not only acts as a chaperone which carries HDAC5 in the cytoplasm in response to CaMKII, but also promotes the trans-phosphorylation of HDAC5 by CaMKII, bringing HDAC5 in proximity to the active site of the kinase (Backs et al, 2008).

The calcium-mediated export of class IIa HDACs is involved in the regulation of many physiological processes, such as myogenesis, hypertrophy and neuronal survival (Bolger and Yao, 2005; Shalizi et al, 2006; Metrich et al, 2010 A). In 2005 Bolger and Yao discovered that the CaMKII-mediated HDAC4 export in the cytoplasm exerts a pro-survival role in granule cells exposed to depolarizing low potassium concentrations (Bolger and Yao, 2005). On the opposite, the pharmacological inhibition of CaMK with KN-93 causes the nuclear accumulation of HDAC4 and the induction of apoptosis (Bolger and Yao, 2005). Further studies clarify that the pro-survival effect associated to HDAC4 nuclear export is due to the activation of MEF2 (Shalizi et al, 2006). Recently it was discovered that in cardiomyocytes the pro-hypertrophic stimuli can be regulated by cAMP via the EPAC (Exchange Protein Directly Activated by cAMP) sensor, which activates a pro-hypertrophic pathway involving PLC, H-RAS and CAMKII that culminates in the cytoplasmic accumulation of HDAC4 (Metrich et al, 2010 B). EPAC is a "guanosine nucleotide exchange factor" of the RAS family of GTPases, which, in response to β-adrenergic stimulation and high cAMP, induces cardiac hypertrophy, concomitantly to the activation of H-RAS. In particular EPAC activates PLC, which cleaves phosphatidylinositol 4,5 bisphosphate to diacylglycerol and inositol triphosphate. The latter causes a release of calcium in the cytoplasm that determines the export of HDAC4 via CaMKII, followed by the activation of MEF2-dependent transcription (Metrich et al, 2010 B). This finding is partially in contrast with a previous report where, in myoblasts, the expression of RAS induces the nuclear re-localization of HDAC4 due to its phosphorylation mediated by Erk1/2 (Zhou et al, 2000).

PKD, a kinase activated by PKC, has recently been associated to class IIa HDACs export during lymphocyte maturation (Matthews et al, 2006) and thymic selection (Dequiedt et al, 2005). Of particular interest is the fact that B-lymphocyte activation following BCR (B cell receptor) engagement induces the activation of PKD1 and

PKD3, which phosphorylate HDAC5 and HDAC7 on classical 14-3-3 sites. As a consequence HDAC5 and HDAC7 accumulate in the cytoplasm inducing chromatin relaxation (Matthews et al, 2006) and the activation of MEF2 transcriptional program (Wilker et al, 2008). In order to abrogate the export of HDAC5, a double knock-out of PDK1 and PDK3 is required as the two kinases are partially overlapping.
PKD is also a regulator of T-lymphocyte thymic selection (Parra et al, 2005). HDAC7 is nuclear in "resting" CD4+CD8+ thymocytes and maintains switched-off MEF2s and in particular the MEF2-dependent transcription of the pro-apoptotic nuclear receptor *Nur77/NR4A1*. This orphan nuclear hormone receptor is one of the main responsible for the negative selection. In response to the activation of the TCR (T cell receptor), PKD1 becomes active and phosphorylates HDAC7, which is exported into the cytoplasm. This export determines the activation of MEF2s and the de-repression of *Nur77* that promotes the cell-death (Parra et al, 2005).
PKD is also an important inducer of cardiac hypertrophy via phosphorylation and export of HDAC5 and the relative de-repression of MEF2 (Vega et al, 2004). As a matter of fact, mice with a cardiac-specific deletion of PKD1 show diminished hypertrophy in response to pressure overload or chronic adrenergic and angiotensin II signaling (Fielitz et al, 2008). Several selective inhibitors of PKD are now under clinical studies for the treatment of malignant cardiac hypertrophy, among which one of the most promising is the Bipyridil PKD Inhibitor (BPKDi) (Monovich et al, 2010). PKD1 seems to be an inducer of hypertrophy also in vascular smooth muscle cells (VSMCs). Here treatment with a hypertrophy inducer, such as angiotensin II, stimulates the phosphorylation of HDAC5 by PKD1 (Xu et al, 2007).
Finally, in 2008 Olson group demonstrated that the exogenous expression of PKD1 in type II skeletal muscle fibers promotes the phosphorylation and the nuclear export of HDAC4 and HDAC5, the subsequent activation of MEF2 TFs and the switch of these fiber from fast/glycolytic into red oxidative (slow-twitch type I) (Kim MS et al, 2008), in accordance to the phenotype observed in transgenic MEF2C-VP16 mice (Potthoff et al, 2008 A).

Another kinase involved in class IIa HDACs re-localization is LKB1. LKB1 regulates and activates two classes of kinases: the MARK kinases (Microtubule Affinity Regulating Kinase) and in particular hPar EMK-1 and c-TAK-1, and the AMPK kinase, that are AMP-dependent (Ling and Mills, 2013).
The MARK kinases phosphorylate class IIa HDACs constitutively on a residue (S159 in HDAC7, S246 in HDAC4, S259 in HDAC5). This base-line phosphorylation facilitates the subsequent signal-dependent phosphorylation by other kinases of the remaining residues required for 14-3-3 binding (Dequiedt et al, 2006). Additionally, Par-1 kinases are involved in the determination of the polarity, both at the systemic level by controlling the polarization of the gastrula in Drosophila (Bayraktar et al, 2006) and the first asymmetric division in C.*elegans* (Guo and Kemphues, 1995), and at a cellular level, by inducing the expression of Delta only along the basolateral epithelial membranes, thus guiding the detachment of migrating cells from the underlying epithelium (Bayraktar et al, 2006). Also in this case the determination of the polarity seems to require the relaxation of the chromatin and, apparently, the abrogation of class IIa HDACs activity.
The AMPK kinase is activated under conditions of metabolic stress and ATP depletion (Liang and Mills, 2013). Once activated, it phosphorylates HDAC4, thus inducing its nuclear export (van der Linden et al, 2007). At a physiological level, even the stress induced by physical exercise determines the export of HDAC4 and 5 via the activation of AMPK and CaMKII (McGee et al, 2009).
The inhibition of LKB1/AMPK kinases and the subsequent nuclear re-localization of class IIa HDACs is a key-event in order to promote, after fasting, the activation in the liver of the gluconeogenesis (Mihaylova et al, 2011). After a meal, the release of insulin stimulates in the hepatocytes and skeletal muscles cells the synthesis of glycogen and in the adipocytes the storage of energy reserves. These responses involve the activation of Akt

and culminate in the phosphorylation and inactivation of PGC-1α and FOXO 1 and 3 (Gross et al 2008; Canan and Brunet, 2008). The FOXO transcription factors in these conditions are also acetylated and exported into the cytoplasm. LKB1 kinases participate in the signaling by inhibiting the gluconeogenesis and activating AMPK (Gross et al, 2008). During fasting, the release of glucagon in the liver activates the gluconeogenesis, at least in part through the inhibition of the LKB1-AMPK kinases (Mihaylova et al, 2011). The inhibition of LKB1/AMPK, and in particular of SIK2, the kinase responsible for class IIa HDACs' export in this context (Wang et al, 2011), causes a massive accumulation of class IIa HDACs in hepatocytes' nucleus and their association with HDAC3 that deacetylates and activates FOXO1/3. FOXO1/3 then stimulate the transcription of two key enzymes of the gluconeogenesis, the glucose-6-phosphatase and the phosphoenolpyruvate carboxy kinase (Mihaylova et al, 2011).

LKB1 kinases also regulate the activation and the expression of SIK1 (Salt Inducible Kinase) that is responsive to high salt concentrations and CREB. Once activated in myoblasts, SIK1 phosphorylates class IIa HDACs thus promoting their export into the cytoplasm (Berdeaux et al, 2007). SIK kinases are also inhibited during gluconeogenesis, similarly to AMPK, and their inhibition correlates with the nuclear accumulation of HDAC4 (Jaitovich and Bertorello, 2010; Mihaylova et al, 2011). In HEK293 cells, SIK1 is not able to promote the nuclear export of class IIa HDACs (Walkinshaw et al, 2013). A screening of 13 kinases of LKB1 family in HEK293 cells demonstrated that MARK1, MARK2, MARK3/C-TAK1, NUAK1/ARK5, NUAK2/SNARK, SNRK, NIM1 and SIK1 have no significant effects on the subcellular localization of HDAC5 in these cells (Walkinshaw et al, 2013). In contrast to SIK1, ectopic expression of SIK2 or SIK3 causes a dramatic cytoplasmic relocalization of HDAC5 and HDAC9 and, at a lesser extent, of HDAC4 and HDAC7, and the export of class IIa HDACs is inhibited by the contemporary activation of PKA (Walkinshaw et al, 2013). For the cytoplasmic re-localization of class IIa HDACs the catalytic activity of SIK2 is required, while the catalytic activity of SIK3 is dispensable (Walkinshaw et al, 2013). Moreover, while SIK2 promotes the nuclear export through the phosphorylation of 14-3-3 consensus sites, SIK3 is effective also on the mutants Ser to Ala in the 14-3-3 binding sites of HDAC4, demonstrating that the SIK3-mediated export is both kinase activity and 14-3-3 independent (Walkinshaw et al, 2013). Finally, while SIK2 causes the de-repression of MEF2 and stimulates myogenesis in C2C12 cells, SIK3 is incompetent towards MEF2 activation (Walkinshaw et al, 2013).

Following the removal of growth factors and specifically of IGF-1 (Insulin-like Growth Factor), or in situations that simulate muscle differentiation, p38 phosphorylates and activates the Mirk/Dirk1B kinase. In C2C12 myoblasts, Dirk1B promotes the transcription of MEF2C targets, such as myogenin. MEF2C activation is not due to the direct phosphorylation of MEF2C, but it is due to the phosphorylation and nuclear export of HDAC4 and 5 (Deng et al, 2005). Conversely, the silencing of Mirk prevents the differentiation of myoblasts (Deng et al, 2005). The residue phosphorylated by Mirk/Dirk1B is not one of the canonical binding sites for 14-3-3 and it is conserved among HDAC4, 5 and 9, but not in HDAC7 (HDAC4: S266, HDAC5: S279, HDAC9: S240) (Deng et al, 2005). Despite in myoblasts the inhibition of Mirk could not compensate the activation and the nuclear export mediated by CaMK, the action of Mirk should be highlighted because: i) it appears to mediate an export that is 14-3-3 independent, and, ii) Mirk is a negative regulator of the cell-cycle by inhibiting Cdk2 via p27 and by destabilizing cyclin D1(Deng et al 2005; Zou et al, 2004).

As 14-3-3 sites phosphorylation promotes the export of class IIa HDACs, it is logical to imagine that dephosphorylation of the same residues favors the nuclear import. The first evidences in supporting this hypothesis dates back 2000, when Grozinger and Schreiber showed that Caliculin A, an inhibitor of the phosphatases PP1 and PP2A, promotes the export of HDAC4 in the cytosol and reduces its interaction with

importin-α. In the same work it was unequivocally demonstrated the interaction *in vivo* between HDAC4 and 5 and 14-3-3 chaperones (Grozinger and Schreiber, 2000). Among the phosphatases, a major role in the regulation of class IIa HDACs import is played by PP2A complex. PP2A is able to bind the amino-terminal portion of the proteins, in correspondence to the *nls1* and dephosphorylate them (Paroni et al, 2008 18045992). Once de-phosphorylated and nuclear, class IIa HDACs may associate with HDAC3 and N-CoR/SMRT, forming a multiprotein complex enzymatically active (Fischle et al, 2002).

In particular, it seems that there is competition between 14-3-3 and PP2A for the binding to class IIa HDACs, since the association with 14-3-3 chaperones prevents the exposure of the phosphorylated serines, and then the interaction with the phosphatase (Parra et al, 2005). There is also competition between 14-3-3 proteins and MEF2 TFs, since both bind to the same N-terminal domain of class IIa HDACs (Paroni et al, 2008). It could be for this reason that a TM mutant of HDAC4, in which the three consensus sites for 14-3-3 proteins are mutated into alanine, is able to bind for a longer time to the chromatin in respect both to the wild-type form and to a nuclear resident form of HDAC4 mutated in the *nes* (L1062A, Paroni et al, 2008).

Two residues are predominantly dephosphorylated by PP2A in HDAC4. The first is the serine 298 and, following the abrogation of such PTM, HDAC4 translocates to the nucleus (Paroni et al, 2008). This site is also targeted by GSK3β during serum starvation in untransformed cells and constitutes the signal for ubiquitylation and degradation of HDAC4 (Cernotta et al, 2011). The PP2A-mediated dephosphorylation may therefore also protect HDAC4 from the nuclear degradation through the UPS (Ubiquitin-Proteasome-System). It is highly probable that the balance between the activities of GSK3β and PP2A was regulated by p38, with the prevalence of PP2A in the case of massive activation of p38 (Junttila et al, 2008).

The second residue of HDAC4 that is dephosphorylated by PP2A could be the serine 246. The PTHrP (ParaThyroid Hormone-related Peptide) represses chondrocyte hyperthrophy through the repression of MEF2 and RUNX2 activities (Kozhemyakina et al, 2009). The influence on MEF2 and RUNX2 depends on PP2A and the subsequent dephosphorylation of HDAC4 at serine 246 (Kozhemyakina et al, 2009). PP2A is sufficient to induce the import of HDAC4, as the inhibition of PP2A with okadaic acid in U2OS results in the cytoplasmic re-localization of HDAC4. PP2A activity does not influence nuclear accumulation of HDAC4 during apoptosis. In fact, the amino-terminal fragment (aa 1-289) generated by caspase cleavage enters the nucleus without requiring the PP2A-mediated dephosphorylation and retains its effectiveness of repression on MEF2 (Paroni et al, 2007). Also HDAC7 is processed by caspases, in this case by caspase 8, in thymocytes CD4+/CD8 + double positive that undergo apoptosis. Here the cleavage generates an amino-terminal fragment that is unstable and, although accumulating in the nucleus, it has not repressive activity against *Nur77* (Scott et al, 2008). In order to repress *Nur77*, it seems to be necessary the myosin phosphatase complex, consisting of PP1β and MYPT1, that is able to dephosphorylate HDAC7 and thus to stimulate its nuclear re-localization (Parra et al, 2007; Parra and Verdin, 2010). In smooth muscles, the myosin phosphatase dephosphorylates the myosin light chain thus inducing muscle relaxation and it is inactivated following the phosphorylation of MYPT1 on threonine 696 (Ito et al, 2004). Among proteins that are able to phosphorylate and inhibit myosin phosphatase on this residue, there are the kinases of the signaling pathway regulated by the GTPase RhoA (Ito et al, 2004), which are also responsible for the nuclear export of HDAC5 (Chang et al, 2004). It is therefore possible that the activation of RhoA could activate MYPT1, thus resulting in the hyperphosphorylation and export of HDAC5.

In 2007 a report linked nitric oxide (NO) and PP2A. In endothelial cells, NO activates PP2A thus inducing the nuclear accumulation of HDAC4 and HDAC5 and the deacetylation of histone tails. To confirm this observation,

it was used the class II HDAC inhibitor MC1568, which recovered the acetylated status of the histones (Illi et al, 2007).

Ataxia telangiectasia is a neurodegenerative disease caused by mutation of the ATM gene. As ATM kinase is able to phosphorylate and inhibit the activity of PP2A, ataxia telangiectasia is characterized by an accumulation in the nucleus of neurons of HDAC4. Here HDAC4 represses MEF2, cAMP-responsive element binding protein (CREB) and induces heterochromatinization and neurodegeneration (Li et al, 2012).

PP1α is another phosphatase capable of mediating the nuclear import of HDAC4; the activity of this phosphatase can overcome the antagonist activating effect of the phosphatase calcineurin on MEF2 (Perry et al, 2009).

Not always the phosphorylation of a class IIa HDAC correlates with its cytoplasmic localization, as already seen for the phosphorylation mediated by Erk1/2. This is the case of PKA that phosphorylates HDAC4 on serines 265 and 266 (278 and 279 in HDAC5, 242 and 243 in HDAC9) and favors class IIa nuclear accumulation and the subsequent repression of MEF2D and myogenesis (Ha et al, 2010). However, since PKA phosphorylates also MEF2D (Du et al, 2008), it is difficult to discriminate between the repressive activity of PKA due to the direct phosphorylation of MEF2 TFs or the nuclear import of a class IIa HDAC. Recently, Olson group demonstrated that in cardiomyocytes the activation of PKA causes the cleavage of HDAC4 between residues 201 and 202 by an unknown protease (Backs et al, 2011). The ammino-terminal fragment generated accumulates in the nucleus and it is competent for MEF2 repression but it is incompetent for SRF repression (Backs et al, 2011). The binding site for PKA is present only in HDAC4 (aa 638-651) among class IIa HDACs and the anti-hypertrophic effect of PKA is sufficient to antagonize the pro-hypertrophic actions of CaMKII, without affecting cardiomyocyte survival (Backs et al, 2011).

This discovery has given new impetus to the study of the subcellular localization of class IIa HDACs. The first results show the existence of other phosphorylation sites in addition to those for 14-3-3. In the case of HDAC5 at least 17 phosphorylation sites have been recently characterized, 13 of which do not represent the consensus for 14-3-3 proteins (Greco et al, 2011). In particular, the phosphorylation of serine 279 is essential to induce the nuclear import of the protein. This residue is conserved among HDAC4, HDAC5 and HDAC9 and it is assumed, therefore, that the same mechanism regulates the "shuttling" of all the class IIa HDACs, with the exception of HDAC7 (Greco et al, 2011). Curiously, this residue has been previously characterized as a residue phosphorylated by Mirk/DirkB, but in this case its phosphorylation causes the nuclear export of class IIa HDACs (Deng et al, 2005). The kinases responsible for HDAC5 Ser279 phosphorylation have recently been identified to be PKA in Cos-7 cells (Ha et al, 2010) and Cdk5 in neurons (Taniguchi et al, 2012). While PKA promotes the nuclear import of HDAC5 which inhibits MEF2-dependent cardiac fetal gene expression and cardiomyocyte hypertrophy, the phosphorylation by CDK5 promotes the nuclear export of HDAC5; cocaine administration activates PP2A and the dephosphorylation at Ser279 which determines the nuclear import of HDAC5 that acts as a repressor of the gene expression regulated by cocaine (Taniguchi et al, 2012).

In 2012, it was demonstrated for the first time the cell-cycle dependent phosphorylation of HDAC4, HDAC5 and HDAC9, but not of HDAC7, in U2OS cells. The phosphorylation on Ser265, Ser278, and Ser242 respectively by the kinase AuroraB allows their re-localization in the mitotic midzone in late anaphase and in the midbody during cytokinesis (Guise et al, 2012). Consequences of the class IIa HDACs phosphorylation and re-localization is the abolishing of the interaction with the NCoR compex, thus limiting at least in part their repressive activity (Guise et al, 2012). The authors proposed a model to explain a putative cell-cycle dependent regulation of class IIa HDACs activities: during interphase class IIa HDACs are normally exported from the nucleus through a 14-3-3

dependent mechanism; before entry in S or in early M phases, they enter the nucleus through a PKA/PP2A dependent mechanism becoming active and repressive; finally, during mitosis they become phosphorylated by AuroraB, they re-localize and they detach from NCoR becoming less active (Guise et al, 2012). It is possible that the nuclear localization of HDAC4, HDAC5 and HDAC9 during the early phases of mitosis is required for allowing the correct heterochromatinization and segregation of the chromosomes (Cadot et al, 2009; Wuelling et al, 2013). As soon as this process is completed and before cytokinesis, they re-localize thanks to AuroraB phosphorylation in order to allow the correct re-assembly of the nucleus (Guise et al, 2012).

As stated earlier, the main regulators of class IIa HDACs export are 14-3-3 proteins. It was long believed that the 14-3-3 proteins facilitated the export of class IIa HDAC by binding to phosphorylated serines; this binding would induce a conformational change in the deacetylases, which would make them able to expose the carboxy-terminal fragment containing the *nes* to CRM1 (McKinsey et al, 2001). A work by Nishino and colleagues in 2008 has demonstrated that instead the 14-3-3 proteins act primarily by slowing down the nuclear import of class IIa HDACs, in particular of HDAC4 (Nishino et al, 2008). In cardiomyocytes, HDAC4 is phosphorylated by both the splicing isoforms, b and c, of CaMKIIδ and this finding demonstrate that the phosphorylation of HDAC4 could occur both in the nucleus (isoform b) and in the cytoplasm (isoform c), and in the first case it is favored the export, in the second the nuclear import is prevented (Li et al, 2011).

Beside 14-3-3-dependent mechanisms of export, there are also systems 14-3-3 independent, like the already mentioned phosphorylation induced Dyrk1B (Deng et al, 2005). Similarly, there are also systems of import PP2A independent, as the already mentioned import of HDAC4 mediated by Erk 1/2 in C2C12 cells stably expressing the oncogenic form of RAS V12 (Zhou et al, 2000), a not well-characterized ERβ-dependent import of class IIa HDACs in hypertrophic hearths (Pedram et al, 2013) or the import regulated by TRX1 (thioredoxin 1) in cardiomyocytes (Ago et al 2008). TRX1 is able to recruit HDAC4 in the nucleus and to inhibit the activity of some pro-hypertrophic factors such as MEF2 and NFAT (Ago et al, 2008). This regulation of HDAC4 "shuttling", albeit tissue-specific, is very interesting as it is mediated through a post-transduction modification, the reduction of two cysteines, not yet studied on detail and that seems to be occurred earlier than the phosphorylation. In the heart, hypertrophy denotes more usually a pathological condition that is therefore markedly different from hypertrophy in skeletal muscles (Backs and Olson, 2006). Cardiac hypertrophy is characterized by an increase in intracellular ROS with relative oxidation of protein disulfide bridges, and a simultaneous increase in the levels of NAPDH oxidase and xanthine oxidase and a decrease of antioxidants (Backs and Olson, 2006). Recently it has been discovered the presence, under oxidizing conditions, of a disulfide bridge between the cysteines 667 and 669 of HDAC4 (Ago et al, 2008). Cysteine 667 is also positioned in the binding site for the "structural"zinc ion, as previously described, which is coordinated by residues H665, H678 and C667. In a reducing environment, the cysteines 667 and 669 are reduced and the zinc ion binds to its binding site, leading to a protein folding that brings the structural ZBD in contact with the *nes*, masking it to the CRM1 and blocking the nuclear export (Ago et al, 2008). In the presence of oxidants, the cysteines 667 and 669 are oxidized, the zinc is no longer coordinated, the *nes* is exposed to CRM1 and the protein is exported into the cytoplasm (Ago et al, 2008). TRX1 is able to attenuate cardiac hypertrophy in part by restoring the cytoplasmic reducing environment. Furthermore, after the binding to TBP-2, TRX1 reduces DnaJB5 (Hsp40), which in this form can interact with HDAC4 and the complex TRX1-TBP-DnaJB5 reduces the disulfide bridge 667-669 of HDAC4. This is sufficient for determining the nuclear accumulation of HDAC4, in spite of the phosphorylation

status of HDAC4, and the repression of the pro-hypertrophy TFs MEF2, NFAT and GATA4 (Ago et al, 2008; Oka et al, 2009).
The nuclear import of class IIa HDACs could also occur as a consequence of a marked decrease in the activity of the kinases that mediate the nuclear export. For example, the inhibition of LKB1 kinases causes the accumulation of HDAC4 in the nucleus of hepatocytes, thus beginning the gluconeogenesis (Mihaylova et al, 2011). Finally, a report suggested that HDAC4 nuclear import is also stimulated by acetylated p53 in response to DNA damage, and this nuclear import causes a cell-cycle arrest with the repression of cyclin B (Basile et al, 2006).

c) other strategies of regulation: caspase-cleavage, ubiquitylation and SUMOylation.

As aforementioned, HDAC4 and HDAC7 can be cleaved respectively by caspase-3 (Paroni et al, 2004) and caspase-8 (Scott et al, 2008); in both cases the cleavage products increase the apoptotic rate (Paroni et al, 2004; Liu F et al, 2004; Scott et al, 2008), but only for HDAC4 the ammino-terminus fragment, generated by the cleavage, is competent for MEF2 repression (Paroni et al, 2004).

Another mechanism to regulate class IIa HDACs activity is their ubiquitin-proteasome mediated degradation.
The first demonstration of a proteasome-mediated degradation of a class IIa HDAC dates back 2004, when Li and colleagues treated HEK293 cells with two non-selective proteasome inhibitors, ALLN and MG132, and found that the treatment stabilized HDAC4, HDAC5 and HDAC7 (Li et al, 2004). Among the three HDACs, as the stabilization was greater in the case of HDAC7, they focused the study on this HDAC and they figured out that HDAC7 is degraded mainly in the cytoplasm after its phosphorylation-mediated export from the nucleus (Li et al, 2004).
The UPS degradation of HDAC7 in the cytoplasm was recently confirmed in a physiological process, during endochondral ossification (Bradley et al, 2014). As discussed further in details, class IIa HDACs negatively modulate endochondral ossification at the stage of chondrocyte hypertrophy by repressing the activity of both RUNX2 (Vega et al, 2004) and MEF2 TFs (Arnold et al, 2007). In their study Bradley and colleagues figured out that HDAC7 is highly expressed in proliferating cells within the growth plate. Postnatal chondrocyte-specific deletion of HDAC7 increases the proliferation rate of chondrocytes because of the activation of β-catenin (Bradley et al, 2014). During chondrocytes maturation, HDAC7 is exported into the cytoplasm where it is degraded by the proteasome and frees the β-catenin. Therefore the stimulation of HDAC7 degradation could be a promising strategy to promote the expansion and regeneration of cartilage tissues (Bradley et al, 2014).
In 2007, Potthoff and colleagues, studying the switch of the muscular fibers induced by MEF2C (Potthoff et al, 2007 A), demonstrated the proteasomal-mediated degradation of HDAC4 and HDAC5 *in vivo* in murine muscles. Contrary to what it has been previously shown about HDAC7, the degradation of these class IIa HDACs takes place in the nucleus (Potthoff et al, 2007 A). The nuclear degradation of class IIa HDACs was confirmed in 2011 in not transformed cells exposed to serum starvation (Cernotta et al, 2011). In this case GSK3β phosphorylates HDAC4 on serine 298 and this phosphorylation acts as a priming event required for its ubiquitylation and nuclear degradation (Cernotta et al, 2011).
Recently, it has been demonstrated that the stimulation of osteoblast differentiation with PTH causes the phosphorylation of rat HDAC4 on serine 740, its export in the cytoplasm and its degradation through a system that is proteasomal-, caspase 3-, serine- and aspartic- proteases independent but lysosomal dependent (Shimizu et al, 2014). If confirmed these data could suggest the existence of a lysosomal pathway of class IIa HDACs degradation.

Class IIa HDACs are also subjected to SUMOylation (Kirsh et al, 2002). In particular, HDAC4 becomes SUMOylated on lysine 559 by the SUMO E3-ligase RanBP2 on the nucleopore complex during its nuclear import (Kirsh et al, 2002). The conjugation of SUMO-1 to HDAC4 does not require any priming phosphorylation signal. The SUMOylation increases the interaction of HDAC4 with HDAC3 and therefore its repressive capability (Kirsh et al, 2002). Also HDAC5 and HDAC9, but not HDAC7, are similarly SUMOylated respectively on lysines 605 and 549 (Kirsh et al, 2002); the lack of HDAC7 SUMOylation is probably due to the absence in HDAC7 of the glutamine rich-self aggregating region (Guo et al, 2007). In Parkinson disease (PD), it is reported that the ubiquitin E3-ligase Parkin ubiquitylates and stimulates the degradation of RanBP2, thus decreasing the SUMOylation of HDAC4 (Um et al, 2006); this event could contribute to neuronal cell death in PD (Um et al, 2006).
Class IIa HDACs not only are targeted by the SUMO E3-ligases, but several evidences demonstrate that they could induce the SUMOylation of some of their targets and for many years they were considered having a SUMO E3-ligase activity. Nowadays it has been clarified that class IIa HDACs have no SUMO E3-ligase activity (Gregoire et al, 2006), but they are involved in the activation of the SUMO E2-ligase Ubc9 (Gregoire et al, 2006). In this way class IIa HDACs promote the SUMOylation of MEF2s (Gregoire et al, 2006), PML (promyelocytic leukemia protein) (Gao et al, 2008), the nuclear receptors LXRα/NR1H3 and LXRβ/NR1H2 thus stimulating their binding to STAT1 and the inhibition of an inflammatory response (Lee JH et al, 2009).

5. Partners and biological functions

Class IIa HDACs mainly exert the function of transcriptional co-repressors (Wang et al, 1999). However, the study of class IIa HDACs' biological functions faces significant technical difficulties:
I) at least in cultured cells class IIa HDACs can act promiscuously to repress gene transcription; this redundancy among the four class IIa HDACs makes extremely difficult the interpretation of the single knock-down experiments (Chang et al, 2004; Haberland et al, 2009; Mihaylova et al, 2011; Clocchiatti et al, 2013 B).
II), in some cellular models the silencing of a single class IIa HDAC induces the compensatory up-regulation of the others (Mihaylova et al, 2011; Clocchiatti et al, 2013 B).
III) being enzymatically inactive, the HDAC activity could not be used as a read out of their activity (Fischle et al, 2002) and after any experiment of interference the residual activity of a class IIa HDAC should be indirectly quantified by measuring the transcriptional activity of one of its TFs partners.
iv) at least in the case of HDAC4, because of the sensitivity of the C-terminus to redox conditions (Ago et al, 2008), researchers should be extremely cautious in verifying the redox status of the medium in which they are growing the cells and studying the activity of HDAC4.

Powerful tools for the *in vivo* studies of the processes regulated by class IIa HDACs are the knock-out (KO) mice. However, considered the aforementioned redundancy and compensatory mechanisms among class IIa HDACs, the single knock-outs could reveal only the most exasperated functions for which there are no strong compensatory mechanisms, or there is no functional redundancy.

Mice HDAC4 KO die within two weeks from birth manifesting early and ectopic ossification (Vega et al, 2004). Mice lacking HDAC5 or HDAC9 are vital, but are characterized by an exacerbated cardiac hypertrophy triggered by hormonal stress-related signals (Chang et al, 2004). Moreover, HDAC9 knock-out mice are exaggeratedly sensitive to the denervation responses in skeletal muscle (Mejat et al, 2005). The loss of HDAC7 is fatal in embryonic age because of copious hemorrhages caused by endothelial dysfunction and loss of joints (Chang et

al, 2006). Combining these *in vivo* studies with *in vitro* observations, there have been identified three key partners of class IIa HDACs: the transcriptional activators MEF2s (Myocyte Enhancer Factor 2), RUNX2 (Runt-Related Transcription Factor 2) and NFAT (Nuclear Factor of Activated T cells) (Martin et al, 2007). The preferred and most studied targets at the moment are undoubtedly the MEF2 TFs. In particular, it is interesting to note that the phenotype of the single knock-outs of class IIa HDACs could be explained as the effect of MEF2 over-activation in bone (HDAC4), hearth (HDAC5/9) and cardiovascular system (HDAC7), in relation to the district in which the single HDACs are more abundant (Vega et a, 2004; Chang et al, 2004; Chang et al, 2006).

Herein, I briefly introduce the most relevant partners of class IIa HDACs:

- MEF2 TFs

The regulation of MEF2 TFs by class IIa HDACs was firstly described in 1999 by Kouzarides group (Miska et al, 1999). The majority of the biological functions of class IIa HDACs are due to the repression of MEF2 TFs activities (Clocchiatti et al, 2011; Clocchiatti et al, 2013 A and B). Class IIa HDACs repress MEF2 transcriptional activity through at least 5 different strategies:

- by recruiting HDAC3/NCoR-SMRT complex and inducing the deacetylation of MEF2 TFs (Gregoire et al, 2007) and of the histone tails in proximity to the promoters of MEF2 regulated genes (Miska et al, 1999; Fischle et al, 2002); however, the ability of the HDAC9 N-terminus splicing variant MITR to successfully repress MEF2 activities demonstrates that the C-terminus of class IIa HDACs is not absolutely required for the repression of MEF2 TFs (Zhang et al, 2001)
- by recruiting the co-repressor CtBP (Zhang et al, 2001)
- by promoting the phosphorylation/dependent SUMOylation of MEF2 TFs (Gregoire et al, 2006), which is repressive “per se” , as explained in the section devoted to MEF2 regulation;
- by competing for the binding to MEF2 with some MEF2 co-activators, such as p300 (Ma et al, 2005)
- by inducing heterochromatinization in proximity to MEF2 regulated promoters (Zhang et al, 2002 A).

Differently from HDAC3 that binds the MADS domain of MEF2 TFs (Gregoire et al, 2007), class IIa HDACs interacts with the MEF2 domain in between aminoacids 39-72 of MEF2C (and corresponding residues in the other MEF2s) (Lu et al, 2000 A) and therefore do not disturb in any way the binding of MEF2 TFs to DNA (Lu et al, 2000 B). Class IIa HDACs do not interfere with MEF2 homo/heterodimerization and the dimerization of MEF2 TFs is not necessary for the binding of the HDACs (Lu et al, 2000 A). The minimal region of HDAC4 required for the binding to MEF2 is a stretch of 18 aminoacids (aa 166-184) that is highly conserved among the four class IIa HDACs and that lies in the hydrophobic glutamine rich-region (Lu et al, 2000 A; Han et al, 2005; Guo et al, 2007). HDAC7, although devoid of the glutamine rich-region, is still able to bind MEF2 as it retains the key residues for the interaction (Dressel et al, 2001). In fact the two residues that are critical for the interaction with MEF2 (leucines 175 and 179 in HDAC4) are conserved among HDAC4, 5, 7 and 9 (Han et al, 2005). Also phenylalanine 178 in HDAC4 (and corresponding residues in the other HDACs) is required for the binding since it fits into a hydrophobic pocket of MEF2; a mutation in this position reduces ten times the binding affinity (Han et al, 2005). The binding between MEF2 TFs and class IIa HDACs takes place both in the nucleus and in the cytoplasm; the interaction with cytoplasmic MEF2 TFs stimulates the nuclear import of class IIa HDACs (Borghi et al, 2001). Class IIa HDACs could bind the DNA only in complex with MEF2 or other TFs (Lu et al, 2000 A); in the case of MEF2 TFs the ternary complex class IIa HDAC/MEF2/DNA forms according to the proportions of 1:2:1 (Han et al, 2005). Interestingly, the interaction of any MEF2 with any class IIa HDACs turns

the MEF2 TFs into transcriptional repressors (Barneda-Zahonero et al, 2013; Clocchiatti et al, 2013 B; Di Giorgio et al, 2013).
MEF2 TFs are the favorite class IIa HDACs partners, as demonstrated by the knock-out experiments. By means of a DNA microarray experiment, in this thesis we demonstrate that among the 49 genes repressed by HDAC4, which constitute the HDAC4 signature, 80% are MEF2 targets. The biological importance of the complex between class IIa HDACs and MEF2s has justified the view of these two distinct families of proteins as a single transcriptional machinery, leading to the concept of the MEF2–HDAC axis (Backs et al, 2011; Clocchiatti et al, 2013 A; Clocchiatti et al, 2013 B).

Class IIa HDACs are able to modulate almost all the biological processes in which the MEF2s are involved (Martin et al, 2007; Potthoff and Olson, 2007). A separate section will be devoted to MEF2s and class IIa HDACs- MEF2 axis functions.

- RUNX2:

RUNX2 belongs to the Runt family of transcription factors (Yoshida et al, 2004). This transcription factor is involved in the regulation of the process of chondrocytes hypertrophy (Zheng et al, 2003; Vega et al 2004) and it is absolutely required for the endochondral bone ossification (Yoshida et al, 2004). Both tumor suppressor and oncogenic properties are associated to RUNX2. In summary, RUNX2 decreases the proliferation of osteosarcoma cells (Lucero et al, 2013) and its depletion is sufficient to escape RAS induced senescence in MEF fibroblasts (Kibery et al, 2007), but it increases the capability of breast and prostate cancer cells to metastasize in the bone (Akech et al, 2010; Javed et al, 2005), and it alters the mammary gland morphogenesis (Owens et al, 2014). Among class IIa HDACs, the main repressors of RUNX2 are HDAC4 and 5, which bind the Runt domain and thus disturbing the DNA binding domain (Martin et al, 2007). HDAC4 and 5 also stimulate the deacetylation of some lysines of RUNX2, determining its proteasomal mediated degradation (Jeon et al, 2006).
Through the repression of RUNX2, HDAC4 blocks chondrocyte hypertrophy and negatively influences the successful completion of endochondral ossification (Vega et al, 2004). The precocious and ectopic ossification of HDAC4 knock-out mice was initially completely explained by Olson group with the activation of RUNX2 (Vega et al, 2004); four years later Olson group partially revised their conclusions demonstrating that much of the phenotype of HDAC4 null mice was actually due to the over activation of MEF2 (Arnold et al, 2007). However, HDAC4 interacts directly with RUNX2; in particular its amino-terminal portion (amino acids 1-220) directly binds the DNA binding domain of RUNX2. As a consequence of this interaction, and differently from MEF2 TFs, RUNX2 loses the ability to bind the promoters to activate (Vega et al, 2004).
The activation of RUNX2 and the subsequent transcription of its target gene VEGF (Vascular Endothelial Growth Factor) are necessary during the process of endochondral ossification for the vascularization step which precedes the differentiation of osteoblasts (Zelzer et al, 2001); HDAC4, through the inhibition of RUNX2, is therefore able to inhibit the expression of VEGF, thus blocking the endochondral bone ossification also at the step of vascularization (Zelzer et al, 2001). In patients with chondrosarcoma, the loss of HDAC4, and the subsequent activation of VEGF and RUNX2, correlates with a poor prognosis (Sun et al, 2009). Finally, HDAC4 and 5 may also be recruited on RUNX2 by Smad3, participating therefore in the TGF-β mediated inhibition of osteoblast differentiation (Kang et al, 2005). HDAC7 is also able to bind RUNX2 and repress its activity, thus stimulating the maturation of osteoblasts; the effectiveness of the repression is not affected by HDAC pan-inhibitors, such as the TSA, thus demonstrating that for the repression of RUNX2 an HDAC activity is dispensable (Jensen et al, 2008).

- NFATc3:

NFATc3 is a transcription factor that activates the genetic program of slow-twitch muscle fibers, in synergy with MEF2 (Potthoff and Olson, 2007). The chaperone DnaJ (MRJ) is able to interact with and repress NFATc3, via recruitment of HDAC4, 5 and 7 (Dai et al, 2005). In the heart NFAT induces hypertrophy, stimulating the expression of some pro-hypertrophic genes such as MYH7, RCAN1, Cx43, Anf and BNP. An antagonist of NFAT is FOXP1, which acts by repressing the same genes (Bai and Kerppola, 2011). During cardiac hypertrophy, CaMKII activation induces the nuclear export of class IIa HDACs and the de-repression of MEF2 TFs and of NFAT (Metrich et al, 2010 A). A correct balance between the activities of NFAT, FOXP1, MEF2 and class IIa HDACs is therefore required for the maintenance of cardiac homeostasis (Bai and Kerppola, 2011).

- CTBP:

The co-repressor CtBP binds its partners thanks to the CtBP-binding motif (P-X-D-L-R), which is present in HDAC1, 3, 4, 5, 7, 9 and MITR, and as a matter of fact, CtBP interacts with all of them (Zhang et al, 2001). At least in part the binding of class IIa HDACs to CtBP is required for a successful repression of MEF2 TFs. In fact, mutation of the CtBP-binding domain in MITR abolishes its interaction with CtBP and impairs, but does not eliminate, the ability of MITR to inhibit MEF2-dependent transcription (Zhang et al, 2001). In order to interact with the DNA, CtBP must be bound to NADH and the oxidation of NADH to NAD+ weakens its activity (Zhang and Arnosti, 2011). Since both CtBP (Zhang and Arnosti, 2011) and HDAC4 (Ago et al 2008) are under the influence of the redox status, it will be important to investigate whether the CtBP-HDAC4 axis acts as a hypoxia responses regulator.

- HP-1α: Class IIa HDACs as regulators of heterochromatinization

There are several evidences about a role of class IIa HDACs in the regulation of heterochromatinization. The regulation of this process by class IIa HDACs is debated and the evidences are still not so convincing.
However, it is important to discuss about a putative role of class IIa HDACs in these processes because:

- heterochromatinization is a well-known strategy to repress genes, especially those that require rapid modulation such as those regulated during embryogenesis (Kehat et al, 2011)

- it would suggest a role of class IIa HDACs in the mechanisms of senescence escape and oncogenesis (Di Micco et al, 2011).

In 2002 Olson group described class IIa HDACs as the molecular link between histone deacetylation and methylation (Zhang et al, 2002 A). In the same years it was demonstrated the enzymatic ineptitude of class IIa HDACs (Fischle et al, 2002); in the following years it was further demonstrated by detailed biochemical and structural studies that these HDACs have no deacetylase activity per se but act as lysine-deacetylases only if in combination with class I HDACs (Lahm et al, 2007). Moreover the restoration of an active catalytic site is completely dispensable for the carrying out of some typical class IIa HDACs functions, such as MEF2 repression (Lahm et al, 2007).

Taking this into account, Class IIa HDACs are proposed regulators of heterochromatinization through two different mechanisms:

- the “classical pathway”, through the recruitment at certain genomic loci of class I HDACs and the class I mediated histone deacetylation

- the “unconventional pathway”, through the recruitment of other factors, such as methylases.

Here I will focus the discussion on the unconventional pathway.

Starting from a yeast-2 hybrid screening using as a bait a natural truncated form of HDAC9 (MITR) that lacks the histone deacetylase catalytic domain, MITR was found to interact with HP-1α. This property is shared among all class IIa members (HDAC4, 5, 9) but not among class I HDACs and the interaction, which is direct, was proved to be effective also at the endogenous level. In particular, two adjacent regions in the N-terminus adaptor region of MITR (aa 390-400 and 400-490) interact with the hinge region of HP1α (aa 68-95). This dimeric complex exists also as a trimer associated to SUV39H1 and also the existence of a dimer containing the HDACs and SUV39H1 is not excluded. The formation of this complex in proliferating myoblasts is believed to participate in MEF2 transcriptional inactivation and as a matter of fact the myogenin promoter is significantly methylated on lysine 9 of H3 in undifferentiated myoblast. The proposed model predicts that class IIa HDACs facilitate the binding of HP-1α to the sites methylated by SUV39H1; this triggers a chain reaction in which adjacent histone sites are methylated one after the other, thus spreading the heterochromatinization (Zhang et al, 2002 A).

The existence of a complex containing HDAC9 and HP-1α suggests a contribution of class IIa HDACs in pericentric heterochromatin formation and maintenance. Pericentric heterochromatin is a formation of heavily packed DNA that surrounds the centromere, made up of satellite repeats and de novo silenced regions of euchromatin (Roldan et al, 2005). These DAPI-dense regions characterized by H3K9 and H4K20 tri-methylation (Lehnertz et al, 2003; Gonzalo et al, 2005) are the result of the combined recruitment on DNA of SUV39H1, HP-1 and Suv4-20h2. They are necessary for the correct chromosome segregation in mitosis (David et al, 2003) but also for quickly and effectively silencing the expression of some genes and any interference in its formation induces cell-cycle arrest in G1/S an G2/M transitions and apoptosis (Sims and Wade, 2011). A similar phenotype was observed after HDAC4 silencing in p53 deficient HeLa cells by Cadot and colleagues (Cadot et al, 2009). In fact after HDAC4 silencing HeLa cells arrested in G2/M because of segregation defects of the chromosomes (Cadot et al, 2009). At that time it was not explained exactly the mechanism of action of HDAC4 in a context p53 -/-. Although data are preliminary and further studies are necessary, a role of class IIa HDACs in heterochromatin formation cannot be excluded.

Ten years after Olson paper, Mottet group demonstrated that HDAC5 is effectively involved in the maintenance of pericentromeric heterochromatin (Peixoto et al, 2012). In particular HDAC5 co-localizes with HP-1α in late S-phase and HDAC5 silencing impairs the capability of HeLa and MCF-7 cells to proliferate and duplicate the DNA. This is not due to defects in licencing complex assembly or DNA wrapping on the histone octamer but to replication fork stalling arising from defects in heterochromatin formation. The stalling of the polymerase causes DSB, the activation of the checkpoint kinase Chk1 and of the cell-cycle regulators p53, p21, p27 and p16 and the apoptotic response. It is extremely interesting that all these defects induce a reversible cell-cycle arrest in HeLa cells and a harsher and less reversible effect, in MCF-7 cells. Authors suggested that in HeLa cells the defects are corrected after the activation and recruitment on DNA damaged sites of the endonuclease Mus81, which could interact with HDAC5 itself. A similar activation probably is impaired in MCF-7 cells due to Mus81 mutations. Another putative explanation to this paradox could be the compensatory activation of others HDACs as a consequence of HDAC5 silencing, a phenomenon that is extremely strong in some contexts (Mihaylova et al, 2011; Clocchiatti et al, 2013 B).

Heterochromatinization is clearly involved in maturation of erythroblasts (Popova et al, 2009). In particular erythroblasts differentiation requires a temporally well-defined heterochromatinization that culminates in nuclear extrusion (Ragoczy et al, 2006; Popova et al, 2009). Using a murine model of erythroblasts maturation

in vitro, the FVA cells (murine erythroblasts coming from the spleens of Friend virus-infected mice), in 2009 it was demonstrated the involvement of HDAC5 in chromatin condensation. In particular the differentiation of FVA cells *in vitro* takes place in 48 hours. During this time a major event is the compaction of chromatin, before the extrusion of the nucleus, but surprisingly this event is not associated with an increase nor of non-histone proteins nor of architectural factors involved in heterochromatinization (HP1, NMEI, MENT, MeCP2, MBD2, macroH2A and H2A.Z), differently from other species (e.g. chicken) or other differentiation models (e.g. myoblasts differentiation). HDAC5 seems to be the crucial factor capable to decrease the acetylation of K12 on H4, thus expanding the region of facultative heterochromatin positive for H3K9me2 that surrounds the pericentromeric heterochromatin area positive for H3K9me3 and that is unchanged during differentiation (Popova et al, 2009). Therefore in this model HDAC5 is associated to facultative and not constitutive heterochromatinization, differently from what seen in cancer cells by Mottet group (Peixoto et al, 2012). However a limitation of Popova paper is that the direct involvement of HDAC5 in chromatin condensation is not demonstrated, because in order to abolish HDAC5 functions they used two pan-inhibitors of class I and II HDACs, TSA and NaB, instead of its silencing. Since these inhibitors strongly inhibit the maturation of erythroblasts, authors emphasized how the use of deacetylase inhibitors for treatment of hematologic malignancies (Claus and Lubbert, 2003), could have possible adverse effects on erythropoiesis.

A possible explanation for the apparent contradictory dual role of HDAC5 as a promoter of the formation of both constitutive and facultative heterochromatin comes from a paper published by Cossart group on the transcription factor Bromo Adjacent Homology Domain–containing protein 1 (BAHD1) (Bierne et al, 2009). This poorly characterized transcription factor seems to act as a tumor suppressor in lung cancer (Brena et al, 2007) and is involved in heterochromatin formation (Bierne et al, 2009). In 3T3 fibroblasts the over-expression of BAHD1 is sufficient to re-localize HP-1 from regions of constitutive heterochromatin H3K9me3 positive to areas of facultative heterochromatin H3K27me3 positive. Authors suggested that in these areas BAHD1 might act as a genomic anchor of factors involved in the spreading of heterochromatin, such as MBD1 and HDAC5 (Bierne et al, 2009). Regarding to HDAC5, exogenous BAHD1 is found to co-immunoprecipitate with endogenous HDAC5 in 293 cells and to bring HDAC5 to the IGF2 promoter, an event that is correlated to the repression of IGF2 transcription. Up to now it is not known if this recrutiment of HDAC5 to IGF2 promoter is directly mediated by BAHD1 or by HP-1 or due to the binding to MEF2s and if HDAC5 has effectively a key-role in the spreading of heterochromatin down to IGF2 promoter. Authors suggested the involvement of EZH2 in the methylation of H3K27 in IGF2 promoter and in the translocation of BAHD1 repressor complex from H3K9me to K3K27me regions. Unfortunately, in the last 5 years no considerable improvements have been made in the characterization of this mechanism.

Recently a detailed comparative study on the promoter status of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in failing and non-falling myocardium (Hohl et al, 2013), further clarifies the involvement of class IIa HDACs in the regulation of methylation and heterochromatinization. It is well known that hearth failure is characterized by the reactivation of some fetal genes, including ANP and BNP (Saito, 2010). Using sections of human healthy or pathological hearts, authors clearly demonstrated that the induction of those genes is due to a decrease in the methylation status of H3K9me2 and 3 rather than an increase in the acetylation status of H3K9, H3K27 and H4K91. This de-methylation in failing hearths is not due to a decrease in the histone methyltransferases (G9a and SUV39H1) and could be only partially explained by a moderate increase in the expression of histone demethylases (LSD1, JMJD1, and JMJD2). Instead, authors demonstrated a direct role of HDAC4 in the regulation of the accessibility of *ANP* and *BNP* promoters in the hearth. In a non-

pathological condition HDAC4 is abundant in the nucleus of cardiomyocites. Instead, in hyperthrophy and in failing hearths, HDAC4 is highly exported in the cytoplasm, through activation of Ca2+ signaling and of CaMKIIδB. If active and nuclear, HDAC4 binds MEF2 and brings a repressor complex made up of SUV39H1 and HP-1α on *ANP* and *BNP* promoters, thus promoting the methylation of H3K9 and gene silencing. If inactive and cytoplasmic HDAC4 allows MEF2 to actively transcribe *ANP* and *BNP*. The importance of HDAC4 role in the control of the methylation status of the promoter of *ANP* is confirmed by the evidence that in mice with cardiomyocyte-specific deletion of HDAC4 the baseline of H3K9 and H3K27 methylation in the ANP promoter region is reduced compared to WT mice. Further evidences that support this model comes from refined studies with isolated working hearts subjected to elevated preload, taken as models of pre-pathological conditions. In this case, an elevation in the pre-load induces the nucleo-cytoplasmic shuttling of HDAC4, the dissociation of HP-1α from the *ANP* promoter and the transcription of *ANP* (Hohl et al, 2013).

Even though this paper portrays a complete study of *ANP* and *BNP* promoters with excellent techniques, at least two questions remain unresolved: how the de-methylation of these promoters takes place (involvement of a not characterized demethylase?) and how could be explained that HP-1α binding to the promoter region of *ANP* decreased in WT mice, but increased in HDAC4-KO mice?

In summary, multiple evidences point towards a role of class IIa HDACs in epigenetic regulation of gene transcription. These HDACs act as transcriptional repressor by stimulating histone tails deacetylation through the engagement of class I HDACs (the classical action of class IIa HDACs) or DNA methylation and heterochromatinization through the recruitment of methylases or HP-1α.
To date, most convincing demonstrations of the involvement of class IIa HDACs in the process of heterochromatinization are required. For example, it would be necessary to show whether the defective HDAC domain has a role in the heterochromatinization and how much of the repressive effectiveness of class IIa HDACs is really due to the induction of heterochromatinization.

However, as anticipated, heterochromatinization has recently been associated with the regulation of many processes, including senescence and embryogenesis.
In particular, d'Adda di Fagagna group suggested in 2011 (Di Micco et al, 2011) that during the initial steps of oncogenesis, cancer cells escape senescence and the proliferative block imposed by DNA Damage Repair (DDR) complexes by inducing the formation of heterochromatin. The demonstration of an involvement of class IIa HDACs in this process will stimulate again the identification of selective class IIa HDACis inhibitors (Di Giorgio et al, 2015).

HDAC4 was identified as a promoter of heterochromatinization also through the association to Nucleoporins (Kehat et al, 2011). It is becoming clear that the nuclear architecture can in some way modulate gene transcription. Gene-rich chromosome territories occupy the interior regions of the interphase nucleus, whereas gene-poor domains are localized to the nuclear periphery in proximity to the nuclear lamina. Similarly, transcriptionally active genes tend to reside closer to the nuclear interior and dissociate from the lamina thanks to Nucleoporins. This distribution is partially subverted during development, when the rapidity required in transcriptional responses causes the formation of chromatin loops containing transcriptionally active genes extend away from compact chromosome territories, toward the center of the nucleus, where pools of different genes becomes transcribed contemporary, forming the so called "transcription factories" (Bartova et al, 2008). Kehat and colleagues discovered that HDAC4, among class IIa HDACs, has the peculiar capability to interact with the Nucleoporin Nup155. As a result of this interaction, HDAC4 prevents the association of certain

genomic regions to this Nucleoporin, thus preventing their activation and transcription (Kehat et al, 2011). Upon certain stimuli, HDAC4 relocalizes in the cytoplasm and in this way the transcription of the genes is reactivated (Kehat et al, 2011).

If confirmed, Kehat results candidates HDAC4 to the role of master regulator of several developmental processes. In addition to its role as regulator of endochondral bone ossification (Vega et al, 2004), recently HDAC4 has been described as a regulator of a set of genes in cortical neurons that are crucial for synaptogenesis and plasticity of synapses (Sando et al, 2012, Ronan et al, 2012). To date in both cases MEF2s repression seems to play a pivotal role, but the involvement of HDAC4 in chromatin structure modifications has not been investigated and could not been excluded.

- JARID1B:

The H3K4 histone-demethylase JARID1B encoded by the gene KDM5b has recently been described as a luminal lineage-driving oncogene in breast cancer (Yamamoto et al, 2014). Among the genes regulated by JARIB1B, curiously some are HDAC4 targets (*CEACAM-1, EDN1, ERRFI1, SEMA-5A*) (Yamamoto et al, 2014). In 2007, it was demonstrated that HDAC4 and JARID1B are co-regulated during mammary gland morphogenesis and that HDAC1, 4, 5, 7, 9 are capable to interact with JARID1B (Barret et al, 2007). The region of class IIa HDACs involved in this binding overlaps with the MEF2 binding domain (Barret et al, 2007). Curiously, both JARID1B and HDAC4 repressive activities are stronger in luminal ER+ breast cancers (Clocchiatti et al, 2013 B; Yamamoto et al, 2014) and it is fascinating thinking about a synergistic way of action of an HDAC and a demethylase.

- TRPS1:

TRPS1 is a multi-zinc-finger nuclear regulator of chondrocyte proliferation and differentiation (Wuelling et al, 2013). Recently it was identified as a binding partner of HDAC1 and HDAC4 in chondrocytes. This interaction increases the deacetylation of histone H3 and it is required for the correct heterochromatinization and segregation of the chromosomes during mitosis (Wuelling et al, 2013). Chondrocytes knocked out for TRPS1 display several defects during pro-metaphase that could be almost completely rescued by HDAC4 over-expression (Wuelling et al, 2013).

- 53BP1 and ATM:

In 2003 it was hypothesized that HDAC4 was part of the DNA-double strand damage repair complex (Kao et al, 2003). In particular it was reported that in response to the double strand DNA breaks HDAC4 was recruited in nuclear foci, and co-localize with 53BP1. This happens both in untransformed cells and in cancer lines of breast cancer, glioblastoma and sarcoma (Kao et al, 2003). The number of foci formed is proportional to the intensity of the ionizing radiation, as well as kinetics of formation and persistence of foci (Kao et al, 2003). The system seems to rotate around the interaction, as demonstrated by immunoprecipitation, between HDAC4 and 53BP1 and the silencing of one of the two proteins determines the loss of the other. In this way the silencing of HDAC4 greatly decreases the number of formed foci and limits the block in G2 in response to DNA damage (Kao et al, 2003). The recruitment of HDAC4 in the nucleus probably occurs via p53. The HDAC4-p53 complex thus formed binds to NF-Y, repressing cyclin B (Basile et al, 2006). Recently, it has been demonstrated that HDAC4 nuclear import is inhibited by ATM, a kinase actively involved in double strand DNA damage repair mechanism, and at least in neurons HDAC4 seems to decrease and not increase the activity of the DNA damage repair complex (Li et al, 2012). The study of the involvement of HDAC4 in the DNA damage repair mechanism is therefore still in its infancy and further studies are required in order to have a more clear picture.

- Huntingtin:

Recently, it has been demonstrated that HDAC4 is able to interact both *in vitro* and *in vivo* with Huntingtin protein through its glutamine-rich region (Mielcarek et al, 2013). *In vivo* in cortical neurons from a murine model of HD (Huntington Disease), HDAC4 interacts only in the cytoplasm with Huntingtin. Its knock-down delayed the formation of cytoplasmic aggregates of Huntingtin, restores the transcription of BDNF and rescues neuronal and cortico-striatal synaptic functions (Mielcarek et al, 2013). As the causative agent of HD should be amyloid formation and HDAC4 has short polyQ tracts in its N-terminus, it could be possible that HDAC4 contributes to Huntingtin self-aggregation through its high predisposition to form aggregates. In fact, from a transcriptional point of view, the knock-down of HDAC4 in cortical neurons of 9 week-age mice causes the regulation of only 32 genes and the biological astonishing effect should not be related to this modest transcriptional regulation (Mielcarek et al, 2013).

- ESSRA:

EStrogen-Related Receptor α (ESRRA) gene has recently been identified as mutated in many cases of anorexia nervosa and bulimia nervosa (Cui et al, 2013). The reported mutations determine a loss of function of ESRRA and a strong decrease in its transcriptional activity (Cui et al, 2014). ESRRA mutations are frequently associated to A786T mutation in HDAC4 and the two proteins interact *in vivo* in mouse cortex. This mutation in the C-terminus of HDAC4 has a not well-known role but it is sufficient to increase the transcriptional repressive activity of HDAC4 towards ESRRA (Cui et al, 2014).

- MTA1 AND YY1:

In 2012 it was seen that the Metastasis-associated protein 1 (MTA1), a well-known clinical marker of metastatic breast cancer (Kang et al, 2014) and a key component of the NuRD deacetylase complex (Xue et al, 1998), is able to interact with HDAC4. In particular a ternary complex of MTA1-HDAC4 and YY1 was found on the promoter of PTEN in MCF-7 cells, where it maintains the transcription of PTEN off (Reddy et al, 2012).

- FOXO AND FOXP3:

Class IIa HDACs acts both as negative and positive regulators of the activity of some member of the large family of FOXO transcription factors (Clocchiatti et al, 2013 A).

In particular, Class IIa HDACs are reported to inhibit FOXP3 functions in Treg cells, acting therefore as immunomodulators (Tao et al, 2007; Zhou et al, 2008). In breast cancer, it has been shown that HDAC4 binds FOXP3 on the first intron of p21 keeping off the transcription of p21 (Liu et al, 2009). On the opposite, as already described, class IIa HDACs brings HDAC3 in contact with FOXO1/3 and stimulate their deacetylation and activation (Mihaylova et al, 2011).

6. Class IIa HDACs as regulators of proliferation and cancer

In the early years of this decade several efforts have been persuaded on the identification, synthesis and characterization of HDAC inhibitors (HDACis). Several HDACis have entered multiple clinical trials principally by virtue of their anti-neoplastic properties (Bolden et al, 2010). These molecules display both cytostatic properties, especially through the induction of p21 and blockage of the cell-cycle (Vrana et al, 1999; Richon et al, 2000), and cytotoxic effects, by triggering apoptosis via multiple mechanisms (Vnrana et al, 1999; Henderson et al, 2003; Gammoh et al, 2012), or by stimulating the clearance from the immune system (Maeda et al, 2000; Magner et al, 2000). Despite the promising anti-neoplastic properties, entering of HDACis in clinic is slower than expected, principally due to some side effects and toxicity displayed during early-phase clinical

trials (Lane et al, 2009), and until now only two HDACis have been approved for the treatment of malignancies: SAHA and Romidepsin/FK-228, both for the treatment of T-cell lymphoma (Balasubramanian et al, 2009). In the last few years increasing evidences about pro-oncogenic roles of class IIa HDACs have encouraged the development of specific inhibitors of this class of HDACs. These molecules, improperly defined inhibitors, do not interfere with the deficient catalytic activity of class IIa HDACs but they act by deconstructing the proteins, or altering their sub-cellular localization or preventing their interaction with certain functional partners (Di Giorgio et al, 2015).

The high expectations around these new molecules have increased the interest in the most neglected class of histone deacetylase, the IIa. To date, in literature there are more than 200 articles that provide direct or indirect evidences of the putative oncogenic functions of these HDACs.

The first demonstration of HDAC4 proliferative effects comes from the phenotype of the knock-out. Mice HDAC4 -/- die because of premature and ectopic endochondral ossification, which determines their suffocation (Vega et al, 2004). This phenotype resembles the tissue specific over-expression of MEF2C and RUNX2 (Vega et al, 2004; Arnold et al, 2007). On the contrary, HDAC4 over-expression slows-down the process of ossification and prevents chondrocytes hypertrophy (Vega et al, 2004). The current model provides that in pre-hypertrophic chondrocytes Indian Hedgehog (IHH) induces the expression of ParaThyroid Hormone-related Peptide (PTHrP), which stimulates the PP2A-mediated nuclear import of HDAC4 (Kozhemyakina et al, 2009) and the expression of Zfp521, a zinc finger transcriptional co-regulator (Correa et al, 2011). Zfp521 mediates the association of HDAC4 with RUNX2 and its repression (Vega et al, 2004; Correa et al, 2011). On the contrary, MEF2C stimulates the expression of *RUNX2*, RUNX2 targets, *Col10a1* and *Caspase-3* with the concomitant repression of *CyclinD1* and *Bcl-2* (Arnold et al, 2007; Correa et al, 2011). Therefore, HDAC4 stimulates chondrocytes proliferation (Vega et al, 2004; Correa et al, 2011) and the fine-tune modulation of Ihh-PTHrP pathway governs the process of ossification (Potthoff and Olson, 2007). The equilibrium is maintained also through miR-365, a miRNA that targets HDAC4 mRNA under mechanosensitive stimuli (Guan et al, 2011). Another level of regulation is offered by HDAC4 subcellular re-localization. In particular, HDAC4 is located in the nucleus of chondrocytes in the proliferation zone of the growth plate and relocates to the cytoplasm of chondrocytes in the pre-hypertrophic zone *in vivo* (Guan et al, 2012). The shuttling between the nucleus and the cytoplasm is mediated, as in the muscular context, by Ca(2+)/calmodulin-dependent kinase IV mediated phosphorylation (Guan et al, 2012). This balance is broken by exogenous stimulation with mitogens. For example, EGFR activation in osteoblasts increased the protein amounts of HDAC4, thus enhancing *RUNX2* repression, osteoblasts proliferation and decreasing the rate of endochondral ossification (Zhu J et al, 2011).

HDAC4 is also an important regulator of NRF2 pathway. In particular in cancers NRF2 is in general up-regulated and *NRF2* expression negatively correlates with prognosis (Singh et al, 2013). Moreover, the loss of NRF2 decreases the expression of HDAC4, resulting in increased expression of miR-1 and miR-206, two miRNAs which transcription is directly abolished by HDAC4. miR-1 and miR-206 target HDAC4 in a feed-back that determines the block of the pentose phosphate pathway and a loss of tumorigenic potential. Conversely, overexpression of miR-1 and miR-206 decreases the expression of metabolic genes and dramatically impairs NADPH production, ribose synthesis, and *in vivo* tumor growth in mice (Singh et al, 2013).

Recently two groups have independently identified in TRPS1 a new partner of HDAC4. TRPS1 is a zinc-finger transcription factor mutated in chondrocytes of patients affected by Tricho-Rhino-Phalangeal syndrome, an autosomal dominant human disorder characterized by dysplastic hair, short stature, and malformation of the

skeleton. Defects in TRPS1 or loss of TRPS1 cause segregation defects during mitosis (Wuelling et al, 2014). This phenotype is strictly related to an increase in histone H3 (Wuelling et al, 2014) or H4 (Wu et al, 2014) acetylation and a decrease of HP-1 binding to chromatin (Wuelling et al, 2014). In particular TRPS1 seems to behave as a positive regulator of histone deacetylation, by acting in two ways: on one hand it induces the expression of HDAC4 (Wu et al, 2014), on the other hand it enhances its activity (Wuelling et al, 2014). Although these studies are still preliminary, the TRPS1-HDAC4 axis could have oncogenic properties and could influence the progression of osteosarcomas (Wuelling et al, 2014) and luminal breast cancers (Wu et al, 2014). The not redundant role exerted by HDAC4 during mitosis was already described in p53-negative tumor cells (Cadot et al, 2009), even though at that time the mechanism was completely unknown.

Class IIa deacetylases act as transcriptional co-repressors when they are localized in the nucleus. However their cytoplasmic localization is not always linked to the loss of activity. In particular in hypoxic conditions, HDAC4, 5 and 7 are capable of binding to HIF-1α in the cytoplasm and determine its transcriptional activation (Kato et al, 2004, Qian et al 2006 and Seo et al, 2009), thus promoting the expression of VEGF and angiogenesis in cancer cells. Class IIa HDACs activate HIF1α both by stabilizing the protein through the removal of the acetyl moieties affixed to HIF-1α by ARD1 acetyltransferase (Qian et al, 2006), and by binding the domain of HIF1α usually repressed by FIH-1 (factor inhibiting HIF), thus preventing its interaction with FIH-1 and promoting the interaction with p300 (Kato et al, 2004 and Seo et al, 2009). A not convincing point is that HDAC4 in order to interact with HIF-1α should re-localize into the cytoplasm. However, it has been recently demonstrated that HDAC4 resides predominantly in the cytoplasm under oxidizing conditions (Ago et al, 2008, Isaacs et al, 2013) that are not fully compatible with the accumulation of HIF-1α. Differently from HDAC4, HDAC7 is not involved in the activation of HIF-1α, but seems to be necessary for HIF-1α mediated repression of cyclinD1 expression (Wen et al, 2010).

HDAC4 behaves as an oncogene in gastric cancer (Kang et al, 2014). In particular HDAC4 represses p21 and the silencing of HDAC4 decreases the proliferation of gastric cancer cell lines by increasing p21 levels and arresting the cells in G1. HDAC4 silencing increases also the rates of autophagy and apoptosis; the silencing of p21 is sufficient to restore the normal proliferation and survival of gastric cancer cell lines, demonstrating that the majority of HDAC4-mediated effects are due to p21 de-regulation (Kang et al, 2014).

In small intestine, HDAC4 is highly expressed in the epithelium of the crypt, which contains stem and proliferative progenitor cells, and much less in the villus, that contains non-proliferating differentiated epithelial cells (Wilson et al, 2008). Its pattern of expression seems to follow a gradient of proliferation (Wilson et al, 2008). In agreement with this hypothesis, silencing of HDAC4 significantly reduces the proliferation of colorectal-carcinoma HCT-116 cells, which accumulate mainly in G2 (Wilson et al, 2008). These effects mostly depend on the capability of HDAC4 to repress Sp1-mediated p21 CDKN1A transcription (Wilson et al, 2008). Both the nuclear localization and the deacetylase activity are required for HDAC4-mediated repression of p21 (Wilson et al, 2008). Mottet and colleagues confirmed these data in ovarian carcinoma cells (IGROV-1), glioblastoma cells (U87-MG), cervical cancer cells (HeLa) and breast cancer cells (MCF7) (Mottet et al, 2009). In all these cell lines HDAC4 silencing is sufficient to up-regulate p21 CDKN1A in a Sp1 dependent and p53 independent manner. However, p53 is required for the transcription of a basal level of p21 and in absence of p53 HDAC4 silencing is almost ineffective. Therefore HDAC4 seems to act rather as a modulator of p21 levels over a p53 background regulation (Mottet et al, 2009). p21 CDKN1A up-regulation mediated by HDAC4 decreases the proliferation of those cancer cell lines that accumulate preferentially in G1, suggesting a defect

in DNA replication (Mottet et al, 2009) and not in mitosis, as reported by Wilson and colleagues (Wilson et al, 2008). Class IIa HDACs proliferative effect in colorectal carcinoma is confirmed by multiple evidences: I) the overexpression of miR-140 in HCT-116 cells determines a slow-down in the proliferative capability of these cells, that is due to p53 and p21 up-regulation partially mediated by HDAC4 protein, but not mRNA, stabilization (Song et al, 2009); II) a group of compounds containing the trifluoroacetil-thiophene group and that selectively target HDAC4 and HDAC6 display strong anti-proliferative properties in HCT-116 cells (Ontoria et al, 2009). HDAC4 was identified as a repressor of p21 expression also in breast cancer cells through the binding to the transcription factor FOXP3 (Liu et al, 2009). In particular HDAC4 and HDAC2 form a complex on FOXP3 and decrease the acetylation of histone H3 in the proximity of the binding of FOXP3 on p21 promoter, 0.2 Kb down-stream to the transcription start site (Liu et al, 2009). FOXP3 -/- mice show ductal hyperplasia, largely due to the repression of p21 (Liu et al, 2009). HDAC4 is absolutely necessary for the repression of the fraction of p21 transcribed by FOXP3. In fact the over-expression of FOXP3 in MCF-7 cells deprived of HDAC4 is not sufficient to up-regulate p21 (Liu et al, 2009).

p21 has a central role also in MEF2 signature. In fact p21 is a target of some MEF2-regulated genes, such as KLF4 (Shum et al, 2013), KLF2 (Wu and Lingrel, 2004) and NR4A1 (Lee SO et al, 2009). Particularly interesting is the regulation of p21 exerted by KLF4. In fact, in general KLF4 behaves as a tumor suppressor; these properties depend largely on the regulation of the transcription of p21 (Rowland and Peeper, 2006). In fact in a context p21 -/- KLF4 turns to proto-oncogene because of its stemness properties (Rowland and Peeper, 2006). In neuroblastoma cells for the first time a feed-back between KLF4 and MEF2 has been described. In this context MEF2A induces KLF4 that in turn induces MEF2A (Shum et al, 2013).

p21 is regulated also by HDAC5 in neural stem cells (Sun et al, 2007). In this context the orphan nuclear receptor TLX recruits HDAC3 and HDAC5 on the promoters of its target genes, including the tumor suppressor genes p21 and PTEN, keeping off their transcription (Sun et al, 2007). Class IIa HDACs seem to be strong oncogenes in brain cancers. In particular HDAC5 and HDAC9 are significantly up-regulated in high-risk medulloblastoma in comparison with low-risk medulloblastoma; moreover, HDAC5 and HDAC9 expression is associated with poor survival and their silencing in neuroblastoma cells decreases cell growth and cell viability (Milde et al, 2010). Similarly, Sun and colleagues recently demonstrated that HDAC5 blocks differentiation and induces proliferation in neuroblastoma cells (Sun et al, 2014). In particular HDAC5 is induced in these cells by N-Myc. HDAC5 induction feeds a feed-back loop by inducing in turn N-Myc; moreover HDAC5 was found in complex with N-Myc as part of a super-repressor complex that selectively repress the expression of a pool of tumor suppressors (Sun et al, 2014).

HDAC4 and HDAC5 behave as oncogenes also in hepatocellular carcinoma (HCC) (Zhang et al, 2010, Feng et al, 2014). In particular, in human hepatocellular carcinoma miR-22 is highly down-regulated and its re-expression suppresses cell proliferation and tumorigenicity of Hep3B and SMMC7721 cells (Zhang et al, 2010). The main target of this miRNA is HDAC4 and the silencing of HDAC4 displays effects similar to miR-22 re-expression (Zhang et al, 2010). The weakness of this study lies in the fact that the centrality of HDAC4 in hepatocellular carcinoma biogenesis is not investigated and that researchers used TSA, a pan class I-II HDAC inhibitor, in order to prove the druggability of HDAC4 in this context. Another important point is that recently MEF2s have been recognized as potential oncogenes in hepatocellular carcinoma (Ma et al, 2014, Yu et al, 2014). Therefore it is important to clarify whether the oncogenic action of HDAC4 in this context is due to the repression of MEF2s, thereby turning them into repressors, or to the targeting of some other yet unknown targets. The first one is

the more attractive hypothesis and could explain why in their paper Ma and colleagues attribute to MEF2C the function of transcriptional repressor (Ma et al, 2014), as further discussed in the following section. Another miRNA is involved in HDAC4 destabilization in HCC is miR200a. miR-200a was identified for its anti-proliferative properties in HCC cells, both *in vitro* and *in vivo*. The main target of this miRNA is the 3'UTR of HDAC4; HDAC4 itself negatively regulates the transcription of this miRNA, by repressing the transcription factors Sp1. As a consequence of HDAC4 destabilization, miR200a induces the acetylation of p21 CDKN1A promoter and its transcription (Yuan et al, 2011). Regarding the second hypothesis, recently HDAC5 has been described as an oncogene in HCC. Its protein levels are increased in human HCC tissues and in human HCC cell lines. In addition, over-expression of HDAC5 promotes HCC cell proliferation that is on the contrary decreased after HDAC5 knock-down. The proposed oncogenic function of HDAC5 is due to the induction of Six1 expression (Feng et al, 2014), a developmentally regulated transcription factor recently recognized as a strong oncogene, for its capability to induce the expression of cyclinD1 and VEGF (McCarthy, 2012). The results obtained by Feng and colleagues have been partially confirmed by another Chinese group headed by Chen (Fan et al, 2014). Working in depletion, Fan and colleagues demonstrate that in HepG2, Hep3B, and Huh7 cells the knockdown of HDAC5 inhibits cancer cell proliferation by inducing cell-cycle arrest and apoptosis (Fan et al, 2014). These phenotypes are obtained through the up-regulation of p21, the down-regulation of cyclin D1 and CDK2/4/6 and the dysregulation of apoptotic process regulators, such as p53, Bax, Bcl-2 and Caspase-3.

Interestingly in HCC samples, both HDAC4 and HDAC5 can act as oncogenes and single knock-down of each of these two proteins is sufficient to get a phenotype. These results suggest that there is no redundancy. In other context instead a redundancy among class IIa HDAs is reported (Mihaylova et al, 2011, Clocchiatti et al, 2013 B). A possible explanation to this paradox is that the two proteins, although similar and often co-regulated, act in this context on different targets, even though with a similar pro-oncogenic final effect.

Class IIa HDACs have a proliferative function in breast cancer cells, especially in ER+ breast cancers (Duong et al, 2008; Clocchiatti et al, 2013 B). High levels of class IIa HDACs correlates in ER+ breast cancers with a reduced survival (Clocchiatti et al, 2013 B). In MCF7 ER+ cells the inhibition of class II HDACs with specific inhibitors (MCC1568 and MC1575) determines a decrease in the proliferation, the induction of ERb and of p21/CDKN1A and the accumulation of such cells in G1 (Duong et al, 2008). These effects are less pronounced in ER- MDA-MB-231 cells (Duong et al, 2008). In both the cases class II HDACs inhibition does not promote apoptosis (Duong et al, 2008). The triple silencing of HDAC4, HDAC5 and HDAC9 determines a decrease in the proliferation of MCF7 cells (ER+, luminal) but not of MDA-MB-231 ER- cells (triple negative, basal-like) (Clocchiatti et al, 2013 B); however the silencing does not affect the cell-cycle profile (Clocchiatti et al, 2013 B). The anti-proliferative effect of the triple silencing is due to the induction of apoptosis that is dependent on Nur77 up-regulation (Clocchiatti et al, 2013 B). The silencing effects could be mimicked by a class IIa specific HDAC inhibitor that differently from MC1575 is capable to determine the cell death of ER+ breast cancer cells (Clocchiatti et al, 2013 B). Importantly the inhibitor used by Clocchiatti and colleagues (Clocchiatti et al 2013), but not MC1575 (Duong et al, 2008), promoted the up-regulation of certain MEF2-target genes.

HDAC4 determines also the resistance of breast cancer cells to 5-FU, through the deacetylation of the SMAD4 promoter and the repression of its transcription (Yu et al, 2013). In support of an oncogenic role of HDAC4 in luminal breast tumors, the promoter of HDAC4 is unmethylated in poor prognosis luminal breast cancer and methylated in low grade tumors; such correlation between the methylation status of HDAC4 promoter and the prognosis is absent in basal-like breast cancers (Kamalakaran et al, 2011). Similarly, HDAC4 and HDAC9

promoters are hyper-methylated in IGHV non mutated Chronic Lymphocytic Leukemia samples, while are hypo-methylated in IGHV mutated ones (Cahill et al, 2013). The oncogenic potential of HDAC4 in breast cancer is also due to the capability of HDAC4 to repress p21 CDKN1A expression through its binding to the first intron and the induction of deacetylation of histone H3 together with HDAC2 (Liu et al, 2009). This site in absence of HDAC4 is bound by the transcription factor FOXP3 that induce the transcription of p21 CDKN1A and therefore acts as a tumor suppressor in breast cancers (Liu et al, 2009). It is interesting to note that this identified site of HDAC4 binding is completely different from the previously identified in colorectal-carcinomas (Wilson et al, 2008; Mottet et al, 2009).

Class IIa HDACs seem to exhibit oncogenic properties in some gynecological cancers, such as endometrial and ovarian cancers (Ahn et al, 2010; Nakayama et al, 2010). The weakness of these studies is that most of the data were obtained with aspecific inhibitors. The treatment of human endometrial cancer cells with the pan HDAC inhibitor ampicidin displays strong anti-tumor effects, determining the block of cell-cycle and the induction of apoptosis. These effects are mainly due to HDAC3 and HDAC4 down-regulation after the administration of the drug, but it is not possible to discriminate between the effects due to HDAC3 or HDAC4 de-regulation (Ahn et al, 2010). However, HDAC4 has a role in ovarian cancer that is clearly independent from HDAC3 (Stronach et al, 2011). In fact, in ovarian cancer cells resistant to cisplatin, HDAC4 emerges as an activator of STAT1 (Stronach et al, 2011). In particular, resistant cells overexpress both HDAC4 and STAT1 and their depletion is sufficient to re-sensitize cells to the drug. By contrast no effect is reported on sensitive cells. Since the double silencing of HDAC4 and STAT1 has no additive effect in terms of cell death in resistant cells, Stronach and colleagues concluded that HDAC4 and STAT1 act in the same pathway (Stronach et al, 2011). In particular, HDAC4 interacts with STAT1 only in resistant cells. Under these conditions HDAC4 deacetylates STAT1 thus promoting its phosphorylation and nuclear import, which results in a protective response, thanks to the stimulation of IRF1. HDAC4 down-regulation causes STAT1 acetylation and decreases its phosphorylation. Interestingly, deacetylation of STAT1 seems to occur in the cytoplasm and seems to be completely independent from HDAC3 (Stronach et al, 2011).

In lymphoma, HDAC4 has been identified as a target of Mir-155. Ectopic miR-155 expression in mice B cells induces pre-B-cell proliferation followed by high-grade lymphoma/leukemia (Sandhu et al, 2012). Mir-155 acts by targeting the mRNA of HDAC4 and the transcriptional repressor BCL-6 (Sandhu et al, 2012). As a consequence some BCL-6 target genes became de-repressed, such as interleukin-6 (IL6), cMyc and Cyclin D1.

The interest about HDAC7 in Oncology was originated in relation to its ability to regulate vasculogenesis and angiogenesis (Mottet et al, 2007). As evidenced by the phenotype of HDAC7 -/- mice (Chang et al, 2006) that is described in details in the following section, HDAC7 is required during embryogenesis for maintaining vascular integrity (Chang et al, 2006). In particular among class I and class II HDACs, HDAC7 was unique because only HDAC7 specific knock-down impairs human endothelial cell tubulogenesis *in vitro* (Mottet et al, 2007). In particular HDAC7 knock-down prevents the formation of capillary-like structures by HUVECs in type I collagen gel matrix because HDAC7 silencing reduces the chemotaxis of HUVECs, without affecting cell proliferation or apoptosis (Mottet et al, 2007). The reduced migration rate of HDAC7 silenced HUVEC cells is due to the increase in the transcription and secretion of PDGF-B, which alters the cytoskeleton because of a partial inhibition of PI3K/Akt pathway (Mottet et al, 2007). The mechanism that determines the increase in the transcription of PDGF-B after HDAC7 silencing is still unknown. However, the direct involvement of HDAC7 in the process is demonstrated by the fact that PMA administration determines a similar up-regulation of PDGF-B,

which is largely due to HDAC7 nuclear export (Mottet et al, 2007). The pro-angiogenic properties of HDAC7 were completely revised a couple of years later by Olson group. Using the same cellular model, they demonstrated that VEGF induces the PKC/PKD mediated nuclear export of all class IIa HDACs (Wang et al, 2008). Any interference in this VEGF-mediated nuclear export of class IIa HDACs represses endothelial cell proliferation and migration in response to VEGF. In fact, when nuclear, HDAC7 binds MEF2 and a still unknown partner and abrogates the transcription of some early VEGF response genes implicated in angiogenesis in a both MEF2-dependent and MEF2-indipendent manner (Wang et al, 2008). Actually, HDAC7 involvement in oncogenesis is restricted to the regulation of two pathways: c-MYC and beta-catenin. Regarding c-MYC, It is reported that HDAC7 behaves as an oncogene in MCF-7, HeLa and HCT-116 cells. In particular in these cells HDAC7 induces the transcription of c-MYC by promoting the association of RNA pol II with the promoter and its silencing determines a G1/S cell-cycle block and the appearance of senescence. (Zhu C et al, 2011). Margariti and colleagues figured out that both HDAC7 over-expression and HDAC7 knock-down in HUVEC cells prevent the G1/S phase transition, decreasing the proliferation rate (Margariti et al, 2010). In particular, HDAC7 over-expression suppresses endothelial cells proliferation through retention of β-catenin in the cytoplasm and down-regulation of cyclin D1 (Margariti et al, 2010). On the contrary, HDAC7 silencing enhances the nuclear translocation of β-catenin, increases Rb levels and results in enlarged hypertrophic cells (Margariti et al, 2010). This already complex mechanism that recognizes a cytoplasmic function for a class IIa HDAC, is further complicated by the VEGF-mediated degradation of HDAC7. Even not directly explicated by authors, β-catenin could be the mysterious transcription factor, other than MEF2, repressed by HDAC7 under stimulation with VEGF and not identified two years before by Olson group. Hui and colleagues explain the contradictory results obtained by Margariti (the anti-proliferative effect of both the knock-down and the over-expression of HDAC7) with the "fundamental differences in the spatial and temporal properties of HDAC7 between the experiments". In fact while the repressive effect of VEGF on HDAC7 spans minutes and determines a bust in the proliferation of endothelial cells, HDAC7 knockdown produces a prolonged deficit. "Therefore, whereas short-term HDAC7 inhibition results in immediate β-catenin-induced proliferation, a chronic absence of HDAC7 could produce an opposite effect" (Hui et al, 2010). Finally, in 2011 Zhou and colleagues provided the final model of HDAC7 action in endothelial and vascular smooth muscle cells (VSMCs) (Zhou et al, 2011). In particular they demonstrated the existence of two isoforms of HDAC7, a mature spliced and an immature unspliced one, which is 57 base pairs longer (Zhou et al, 2011). HDAC7 is normally maintained as an unspliced isoform in the cytoplasm, where it binds to β-catenin, prevents its nuclear translocation and keeps VSMCs quiescent. After PDGF stimulation, HDAC7 is spliced and is no longer capable of β-catenin binding. The released β-catenin translocates to the nucleus and binds to TCF to activate gene expression and stimulate cell proliferation (Zhou et al, 2011). Therefore this model conceals both Margariti and Mottet evidences, while it discredits Hui hypothetic model. Recently, it has been demonstrated that the HDAC7-β-catenin pathway is also working in chondrocytes (Bradley et al, 2014). In particular HDAC7 knock-down in growth plate chondrocytes increases their proliferation rate; this effects is due to the activation of β-catenin pathway with the induction of cyclin D3 and the repression of p21 CDKN1A (Bradley et al, 2014). The induction of chondrogenesis, with two potent inducers such as ITS and Igf1, determines the export of HDAC7 in the cytoplasm where it is proteasomal degraded. As a consequence β-catenin becomes active (Bradley et al, 2014). In hypertrophic chondrocytes HDAC7 is not detectable probably because of VEGF mediated degradation, in accordance to Margariti model (Margariti et al, 2010). While the final effect of HDAC7 knock-down is similar to the phenotype reported in endothelial cells (Margariti et al, 2010; Zhou et al, 2011), there is a big difference

in the two models: while in HUVECs the interaction between β-catenin and HDAC7 takes place in the cytoplasm and therefore before β-catenin activation (Margariti et al, 2010), in chondrocytes the two proteins interact in the nucleus (Bradley et al, 2014).

The framework around the regulation by HDAC of the β-catenin signaling pathway is complicated by the fact that recently also MEF2 was included in the scenario (Bai et al, 2014). In particular it was demonstrated that in hepatocellular carcinoma (HCC) cells, MEF2C interacts in the cytoplasm with β-catenin and prevents its activation, acting similarly to HDAC7 (Bai et al, 2014). Upon VEGF activation, MEF2C becomes active both because of phosphorylation mediated by PKC/p38 and of its nuclear re-localization, through a still undefined mechanism (Bai et al, 2014). The silencing of MEF2C activates β-catenin signaling and the expression of its targets, such as CyclinD1 and c-MYC and increases the proliferation of HCC cancer cells (Bai et al, 2014). MEF2C seems also able to repress β-catenin transcription (Bai et al, 2014). This effect could be due to the recruitment on β-catenin promoter of HDAC5, as previously seen in murine multipotent mesenchymal cells (Zhao et al, 2011). On the contrary, MEF2C is down-stream to VEGF and positively regulates vasculogenic mimicry and angiogenesis, through the direct transcription of some angiogenic genes, such as MMP9 and TIMP2 (Bai et al, 2014). As a matter of fact tumors in which MEF2C is over-expressed are smaller and less positive for Ki67 in respect to the control but extremely rich in blood vessels and potentially more invasive (Bai et al, 2014).

Class IIa HDACs may play a role also in senescence regulation. In particular class IIa HDACs may promote the senescence escape and the first steps of the oncogenic transformation. It is reported that HDAC9 is up-regulated in IMR90 RAS senescent cells, but the function of this up-regulation is not known (Mason et al, 2004). In human lung fibroblasts 2BS, the acetylation and activation of the transcription factor HBP1 is sufficient to induce p16INK4 transcription and premature senescence (Wang et al, 2012). As TSA treatment abrogates this effect, Wang and colleagues hypothesized that some HDACs could abrogate HBP1 mediated cell-cycle arrest (Wang et al, 2012). Among the HDACs, only HDAC4 is able to bind HBP1 and abolishes its p300 mediated acetylation (Wang et al, 2012). In this way HDAC4 is able to represses HBP1-induced p16INK4A expression, thus repressing HBP1-induced premature senescence (Wang et al, 2012). On the contrary, HDAC4 knock-down enhances HBP1-induced p16INK4A expression and HBP1-induced premature senescence (Wang et al, 2012). In spite of these initial demonstrations, in literature there is still no evidence of a clear involvement of class IIa HDACs in the early stages of oncogenic transformation and in particular in the escape from senescence. As previously discussed, class IIa HDACs may have a role in heterochromatinization and senescence escape (Di Micco et al, 2011). Moreover, as anticipated, in neurons MEF2-HDAC4 axis acts as regulator of the mechanism of DSBs repair (Li et al, 2012; Chan et al, 2014). It is not unreasonable to assume that the phenotype of BJ fibroblasts expressing the combination RAS + HDAC4 may be similar to that of BJ cells expressing RAS + shATM, which, as reported by d'Adda di Fagagna group, is the minimal combination necessary to escape senescence (Di Micco et al, 2006).

Finally, in spite of these reported pro-oncogenic roles, there are also some demonstrations of tumor suppressor roles of class IIa HDACs that sometimes are difficult to reconcile with the first. For example, the over-expression of HDAC5 inhibits the proliferation of U2OS osteogenic sarcoma cells, SY5Y neuroblastoma cells, and MCF7 breast carcinoma cells (Huang et al, 2002). These tumor-suppressor roles are largely due to the activation of tumor necrosis factor receptor pathway and the induction of spontaneous apoptosis (Huang et al, 2002). On the contrary, recently HDAC5 was identified as a promoter of osteosarcoma progression by up-regulation of TWIST1 expression, through a still unknown mechanism (Chen et al, 2014).

Also the quantification of class IIa HDACs cDNA levels in human cancers provides contradditory information: for example HDAC5 and HDAC7 have been founded activated and over-expressed in glioblastomas, where HDAC9 is highly repressed in these tumours (Clocchiatti et al, 2011).

Recently, a mutation in serine 155 of HDAC7, a binding site for 14-3-3 proteins, has been recently described in non-Hodgkin lymphoma (Morin et al, 2011). As a consequence of this mutation, HDAC7 should accumulate in the nucleus of these lymphoma cells and acquire a gain-of-function property. To date, this was the only reported case of a mutation impacting on class IIa HDACs nuclear export in cancer cells.

This brief summary on the most convincing evidences of the involvement of class IIa HDACs in oncogenesis demonstrates how literature is very heterogeneous and confused on the topic and how it is very difficult to define a unique signaling pathway in which class IIa HDACs are involved and whether or not its dysfunction can lead to oncogenic transformation.

7. MEF2 family of transcription factors

Saccharomyces *cerevisiae*, Drosophila and Caenorhabditis *elegans* possess a single Mef2 gene, whereas in vertebrates there are four MEF2 transcription factors (MEF2A-D). These four genes are located on different loci, in humans respectively on chromosomes 15q26, 19p12, 5q14 and 1q12-q23 (Hobson et al, 1995). In agreement with their different genomic location, *MEF2* genes comes from a unique progenitor-seeding sequence that underwent three round of duplication (Wu et al, 2011); the first two events seem to be concomitant to the 2 big duplication events during Gnathostomata and Agnatha divergence (Kasahara, 2007). MEF2B is the most distant from the other three MEF2 proteins in vertebrates, and MEF2C and MEF2A are more closely related. They diverge only from the third duplication event occurred near the origin of vertebrates (Wu et al, 2011, Figure 6). The mutation rates of the four *MEF2* are extremely low, this means that they are under a strong purifying selection, with the exception of MEF2B that evolved faster than the other three MEF2 proteins (Wu et al, 2011).

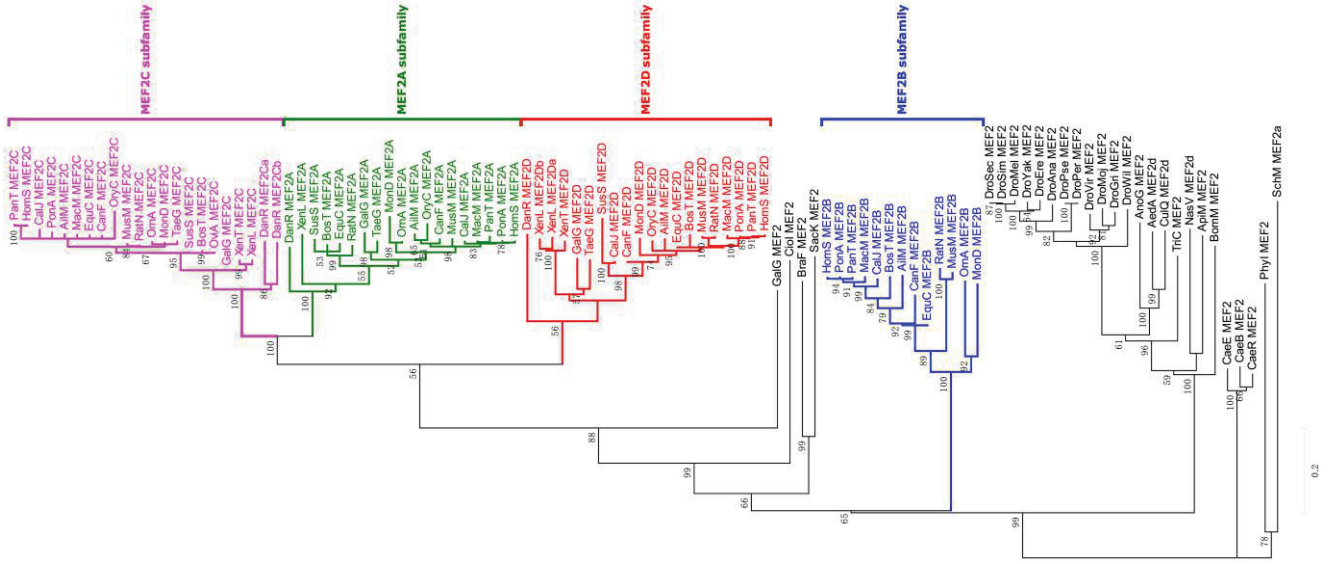

Figure 6 Phylogenetic analysis of the MEF2 transcription factors (adapted from Wu et al, 2011).

MEF2s transcription factors are characterized by a classical bipartite module, typical among the transcription factors. At the N-terminus there is the DNA binding-domain (DBD), while at the C-terminus there are two transcription activation domains (TAD) and a nuclear localization signal (NLS) (Potthoff and Olson, 2007). The DBD is made up of two parts: a MADS box (aa 1-56 in MEF2C), which is named after MCM1, Agamous, Deficiens, and SRF and is directly involved in DNA binding, and a MEF2 domain (aa 57-86 in MEF2C), typical of the family, that mediates the homo and hetero-dimerization between the various MEF2 members and the interaction with co-activators and co-repressors (Potthoff and Olson, 2007). In between TAD1 (aa 143-174 in MEF2C) and TAD2 (aa 247-327) there is a still not characterized inhibitory domain (aa 175-198) (Wu et al, 2010). MEF2s bind the DNA as dimers (Figure 8A) stacked head-to-head with each other (Figure 8B) and recognize the consensus sequence YTA(A/T)4TAR (Andres et al, 1995). A MEF2 truncated after the MEF2 domain and deprived of TADs acts as dominant negative and is sufficient to repress MEF2 transcriptional program (Molkentin et al, 1996 A; Okamoto et al, 2002). While the N-terminus is conserved among the four MEF2 members, the C-terminus is subjected to intense alternative splicing and is extremely divergent among family members (Figure 7), with the exception of the last 35 residues where lies the NLS (nuclear localization

signal) (Yu, 1996; Potthoff and Olson, 2007). A truncation at the level of the NLS prevents the nuclear localization of MEF2 and inactivates its transcriptional activity (Yu, 1996).

Figure 7 Sequence conservation of human MEF2 TFs. In figure in yellow it is indicated the MADS domain (DNA binding), in orange the MEF2 domain (dimerization and interaction with activators and repressors) and in gray the less conserved C-terminus that contains two TADs and a NLS (Adapted from Potthoff and Olson, 2007).

8. On the molecular basis of the MEF2-Class IIa HDACs axis: structure of MEF2/DNA, MEF2/Cabin1/DNA, MEF2/HDAC9/DNA and MEF2/DNA/p300 complexes.

In literature are available: the structure of the MADS/MEF2 domains of MEF2A (aa 2-95) bound to a synthetic double stranded DNA containing the sequence CTATTTATAA (Wu et al, 2010), the crystal structure of the same domains of MEF2A bound to the coactivator p300 (He et al, 2011) and the structures of MEF2B bound to the co-repressors Cabin1 (Han et al, 2003) and HDAC9 (Han et al, 2005).

The structure of each MEF2 monomer consists of an extended N-terminal tail, three helices (H1, H2, and H3) and three beta strands (β1, β2, and β3) (Figure 8C). The MADS domain is formed by: the N-terminal tail (aa 1-15 of MEF2A), helix H1 (15–37), strands β1 (41–49) and β2 (53–59), but only the tail and helices H1 and H3 are involved in DNA binding (Wu et al, 2011). The MEF2 domain is composed by: helix H2 (62–70), strand β3 (76–80), and helix H3 (82–90). This latter is involved in homo- and hetero-dimerization (Wu et al, 2010), in the binding with co-repressors such as Cabin1 (Han et al, 2003) and HDAC9 (Han et al, 2005), of co-activators such as the TAZ2 domain of p300 (He et al, 2011) and of some partners such as MyoD (Wu et al, 2010). The angle of binding between HDAC9 and Cabin1 and MEF2 is different from that between MEF2 dimers; therefore Cabin1 and class IIa HDACs binding to MEF2 do not disturb neither the binding to DNA nor the dimerization (Wu et al, 2010); instead the binding to co-factors and partners is affected (Wu et al, 2010; He et al, 2011). The minor grove (YT *A(A/T)4T* AR) of the DNA consensus sequence is directly bound by aminoacids G2, R3, K4, K5 and I6 of MEF2A; for the binding to the major grove (*YT* A(A/T)4T *AR*) only the tyrosine Y23 is not dispensable, while the positive charged R24, K30, K31 are involved in the interaction with the negatively charged backbone phosphates (Wu et al, 2010). Many residues participate in the dimerization and any mutation in these residues could prevent the correct dimerization (K25, K26, M29, K30, Y33, F55, Q56, S59, E77, S78, T80, N81, I84, V85, L88) (Wu et al, 2010). Helix H3 is instead involved in the interaction with some co-factors, such as MyoD (Wu et al, 2010).

The stable folding of MEF2 domain requires a correct folding of the MADS domain and any mutation in the MADS domain that prevents the DNA binding or alters the structure of the domain causes also the destructuration of the MEF2 domain (Wu et al, 2010). As a consequence, a mutant of MEF2 deprived of its DNA

binding domain is less stable probably because of unfolding of the N-terminus (Di Giorgio et al, unpublished results).

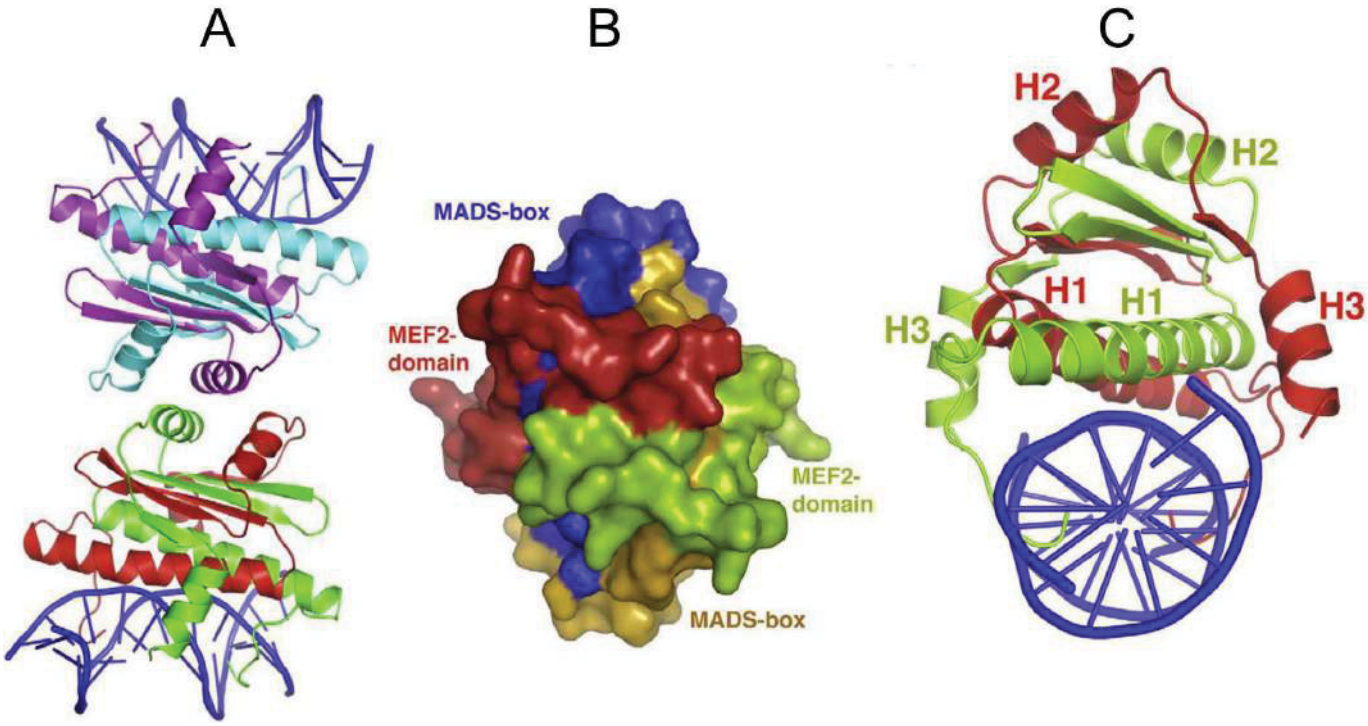

Figure 8 Structure and packing of MEF2A homo-dimer bound to DNA. (A) Representation of two independent MEF2A-DNA complexes stacked head-to-head. (B) Surface representation of a MEF2A homo-dimer. One monomer is in red and yellow, the other in green and blue. (C) Ribbon structure of the same homo-dimer depicted in B, with a view centered on DNA. The alpha-helices of each monomer are depicted with the same color (red or green) (Adapted from Wu et al, 2010).

As anticipated, the MEF2 domain is important not only for dimerization, but also because it mediates the interaction with co-factors and co-repressors. A hydrophobic groove on the MADS-box/MEF2 domain formed by Leu66, Tyr69, and Thr70 and delimited by helix H2 and the flexible linker between H2 and β3 could rearrange to accommodate transcriptional co-activators or co-repressors, such as Cabin1 (Han et al, 2003), class IIa HDACs (Han et al, 2005), MyoD (Wu et al, 2010), p300 (He et al, 2011) and MASTR (Wu et al, 2010). A hydrophobic residue in MEF2 partners (a leucine in HDAC4, HDAC9, Cabin1 and a phenylalanine in MASTR) should insert in the groove in order to mediate the interaction with MEF2 (Wu et al, 2010) and a point mutation in this key residue is sufficient to limit the interaction (Han et al, 2003; Han et al, 2005).

Up to now, among class IIa HDACs, only the structure of HDAC9 bound to MEF2B is available, because of problems of co-crystallization of HDAC4 and MEF2 (Han et al, 2005). The MEF2-binding motif of HDAC9 forms a triple helix interaction with helix H2 of MEF2B on the other face. This interaction does not alter the interactions of MEF2B with DNA and its dimerization properties (Han et al, 2005). The amphipathic helix of HDAC9 binds MEF2 in a manner similar to Cabin1 (Han et al, 2003). Conserved hydrophobic residues on Cabin1 (Thr2168, Leu2172 and Ile2176) and HDAC9 (Val143, Leu147 and Leu151) in fact bind similarly the hydrophobic groove on MEF2B (Han et al, 2003; Han et al, 2005). A big difference between class IIa HDACs and Cabin1 binding to MEF2 concerns a conserved phenylalanine (Phe 150 in HDAC9) that inserts into the hydrophobic pocket in between helix H2 and S3 loop of MEF2. In Cabin1 the corresponding residue is an alanine (Ala 2175)

and the side-chain of alanine is too small to fill the hydrophobic groove of MEF2B and as a result a whole amphipathic helix of Cabin1 folds back and partially fills in the hydrophobic pocket (Han et al, 2003).
The structure of MEF2A bounded to DNA and p300 differs from the previously described because differently from Cabin1 and HDAC9, the TAZ2 domain of p300 binds three dimers of MEF2A/DNA complexes (He et al, 2011). Therefore the complex acquires the shape of a trefoil, with the p300 TAZ2 domain in the center and the MEF2 dimer/DNA complexes at the leaf positions. Despite this big difference, overall the structure of each dimer is quite similar to the previously described (He et al, 2011). Also in this case the interaction with the partner is mediated by helix H2.
To date the main MEF2 partners bind the hydrophobic pocket in between the MADS and the MEF2 domains and for all of them helix H2 is fundamental for the interaction. Therefore it is very difficult to assume that a single MEF2 dimer is capable of binding simultaneously many interactors. At least for the analyzed complexes it seems that the binding to Cabin1 or class IIa HDACs or p300 are mutually exclusive.
Analyzing the well-known partners of MEF2, Wu and colleagues noticed that they are all characterized by a consensus aminoacidic sequence that fits this groove and that constitutes the so-called MEF2 binding sequence, that seems to be a requisite for the binding to MEF2: XX(V/T/I)(K/R)XZ(L/F)ZXX(V/I/L)XXX (Wu et al, 2010).
From these studies on the structure of MEF2 it is evident that the helix H3 is not involved in any interaction neither with DNA nor with co-factors and co-repressors. However, Wu and colleagues showed that also helix H3 is involved in some way in the interaction with DNA (Wu et al, 2011). In particular, Wu and colleagues noticed that the central part of helix H3 is the portion less conserved inside the N-terminus of the four human MEF2 (Wu et al, 2011); moreover, they demonstrated that helix H3 interacts with genomic regions flanking the core of MEF2 consensus region (Wu et al, 2011). It is well-known that, despite the high conservation of the N-terminus, each MEF2 has a preferential consensus-binding (Andres et al, 1995); this consensus is very similar for the four MEF2s, but it highly differs in the regions adjacent to the core (Andres et al, 1995). As the MEF2 signature differs greatly from tissue to tissue, it was proposed, and now almost universally accepted, that the MEF2 isoform more expressed in a determinate tissue is also the one that determines the MEF2 signature. This "leader" MEF2 isoform favors the transcription of genes with the best possible consensus (Andres et al, 1995). Combining these observations, Wu suggested that the specificity of the consensus of each MEF2 depends on the difference between the H3 helix of each MEF2 (Wu et al, 2010).
As an example we reported here the "MEF2 optimal consensus" in three different tissues:

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Muscle N  N  N  N  N  N GT C  T AT AT A  A  A  T  A  G CA N  N  N  N
Hearth N  N  N  N  N TA TG C  T AT AT A  A  A  T  A  G CA N  N  N  N
Brain  N CG  T  G  T  T  A C  T  A AT A  A  A  T  A  G  A AT AC CA N
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9. Pathways of regulation

Historically, the modulation of the transcriptional activity of MEF2s transcription factors was studied in muscle and neurons and most of the available data therefore comes from post-mitotic tissues (Tang et al, 2005; Gregoire and Yang, 2005; Potthoff and Olson, 2007; Magli et al, 2010). Simplifying a very vast and complex literature, there are essentially four processes that can modulate the transcriptional activity of MEF2s:
a) binding to direct repressors, such as class IIa HDACs, HDAC3 and Cabin1 (Miska et al, 1999; Youn et al, 2000 A; Kasler et al, 2000; Lu et al, 2000 A and B; Han et al, 2005; Zhao et al, 2005; Gregoire et al, 2007; Jang et al,

2007); binding to indirect repressors such as Smad3 (Liu D et al, 2004) and sLZIP (An et al, 2014); or coactivators such as p300 (Ma et al, 2005), P-TEFb (Nojima et al, 2008), GRIP-1 (Lazaro et al, 2002), CARM1 (Chen et al, 2002), MAML1 (Shen et al, 2006), ACTN4 (Chakraborty et al, 2006) and Ash2L (Sebastian et al, 2013).
b) post-translational modifications: phosphorylation (Molkentin et al, 1996 A; Kato et al, 1997; Yang et al, 1998; Marinissen et al, 1999; Zhao et al, 1999; Kasler et al, 2000; Kato et al, 2000; Miska et al, 2001; Gregoire et al, 2006; Shalizi et al, 2006; Wang X et al, 2009; Sebastian et al, 2013), SUMOylation (Zhao et al, 2005, Kang et al, 2006, Gregoire et al, 2006), acetylation (Ma et al, 2005, Shalizi et al, 2006, de La Vega et al, 2013), methylation (Choi J et al, 2014).
c) increase or decrease in the transcription of MEF2s and modulation of the stability of the messengers (RNAi) (Black and Olson, 1997; Zhao et al, 2005; Valencia-Sanchez et al, 2006; Chen et al, 2006; Liu et al, 2007 A; Johnnidis et al, 2008; Ikeda et al, 2009; Townley-Tilson et al, 2010; Yelamanchili et al, 2010; Seok et al, 2011; Kalsotra et al, 2014)
d) regulation of protein stability (caspases and proteases mediated cleavages, proteasome degradation and autophagy) (Yu, 1996; Mao and Wiedmann, 1999; Li et al, 2001; Gong et al, 2003; Tang et al, 2005; Butts et al, 2005; Magli et al, 2010).

a) binding to repressors and co-activators

The first demonstration of the binding between a class IIa HDAC and MEF2 dates back to 1999. In that year two English groups shared their data and provided multiple evidences of the binding between class IIa HDACs and MEF2 (Sparrow et al, 1999; Miska et al, 1999). This wonderful example of scientific collaboration provided fantastic data that would be all confirmed by further studies, except for the concept that MEF2 repression exerted by class IIa HDACs required an active HDAC domain (Miska et al, 1999). The interaction between MITR (a N-terminus splice variant of HDAC9) and MEF2A was identified firstly through a yeast-2-hybrid screening using as a bait MEF2A coming from Xenopus and deprived of the glutamine rich region (aa 366-377) and a library of cDNA coming from Xenopus neurula (stage 18) (Sparrow et al, 1999). Miska and colleagues worked on the background data from Sparrow and colleagues and identified HDAC4 as a MEF2A repressor (Miska et al, 1999). Nowadays multiple evidences recognized class IIa HDACs as the main MEF2 repressors. As explained in the previous section, an N-terminal region of class IIa HDACs (aa 166-184 in HDAC4) interacts with a portion of the MEF2 domain of MEF2 proteins; for the interaction the residues VLL (65-67 in MEF2C and corresponding residues in other members) are absolutely required (Molkentin et al, 1996 A). Curiously, the mutation of these residues (VLL65ASR) completely abolishes the transcriptional activity of MEF2 proteins through a still unknown mechanism (Lu et al, 2000 and Di Giorgio et al, unpublished results). Class IIa HDACs do not disturb the DNA binding or the dimerization of MEF2 TFs (Lu et al, 2000), but repress MEF2 activity through three main mechanisms (Clocchiatti et al, 2013 A):
- Recruitment of other co-repressors including class I HDACs and in particular the N-CoR/SMRT/HDAC3 complex (Gregoire et al, 2007)
- Competition for the binding to transcriptional activators, such as p300 (Wang et al, 2012)
- Induction of PTMs such as phosphorylation-dependent SUMOylation (Gregoire et al, 2006).

Another strong repressor of MEF2 is Cabin1, which is under the regulation of the phosphatase Calcineurin and is active mainly in immunological contexts. The interaction between Cabin1 and MEF2B was firstly identified though a yeast-2 hybrid screening (Youn et al, 1999). The C-terminus of Cabin1 interacts with the MADS/MEF2 domain of MEF2B and, as the N-terminus is conserved among the four MEF2, Cabin1 is able to interact with all

the MEF2 (Youn et al, 1999). In double positive thymocytes, TCR engagement led to an increase in intracellular calcium concentration and the dissociation of MEF2 from Cabin1, as a result of competitive binding of calmodulin to Cabin1, with the consequent activation of MEF2B and transcription of the pro-apoptotic gene Nur77 (Youn et al, 1999).

Cabin1 represses MEF2s activities through three main mechanisms:

- the recruitment of mSin3 and its associated HDAC1 and HDAC2 (Youn et al, 2000)
- the competition with p300 for the binding to MEF2 (Youn et al, 2000 B)
- the recruitment of the methyltransferase SUV39H1 on the promoters of MEF2-target genes (Jang et al, 2007).

The structure of the complex Cabin1-MEF2 (Han et al, 2003) was solved before that of the complex Class IIa HDACs-MEF2 (Han et al, 2005), and was used as a model for the further studies on both MEF2 and class IIa HDACs.

Other minor repressors of MEF2s activities have been identified in myoblasts. One of them is the small leucine zipper protein (sLZIP), which prevents the association of MEF2 with the co-activator ACTN4 (An et al, 2014). Also the TGF-beta signaling effector Smad3 could be listed among the MEF2 repressors, as it is able to prevent the binding between MEF2s and the co-activator GRIP-1, thus blocking myogenesis (Liu F et al, 2004).

Among MEF2 activators, the steroid receptor coactivator GRIP-1 and CARM1 (Coactivator-Associated aRginine Methyltransferase) could be found in a ternary complex with MEF2s in differentiating myoblasts and are able to potentiate the activity of MEF2 TFs (Chen et al, 2002). ACTN4 (Alpha-actinin 4) activates MEF2s by competing with HDAC7 for the binding to MEF2A (Chakraborty et al, 2006). P-TEFb (Positive Transcription Elongation Factor b) is responsible for the activation of MEF2-dependent transcription induced by serum starvation. In this condition P-TEFb dissociates from its inhibitory subunit, HEXIM1, and binds, thus enhancing, the transcriptional activity of MEF2s (Nojima et al, 2008). Another co-activator of MEF2 is Ash2L, a methyl-transferase that associates with MEF2D during muscle differentiation and promotes the transcription of MEF2-target genes by inducing the methylation of Histone H3 on lysine 4 (H3K4me3), thus relaxing the chromatin (Rampalli et al, 2007). Finally, in murine myoblasts, the N-terminus (aa 1-70) of Maml1 interacts with MEF2C (aa 87-177) and, by potentiating its transcriptional activity, it positively regulates differentiation (Shen et al, 2006).

b) post-translational modifications

MEF2 TFs are phosphorylated on multiple residues, and in general phosphorylation determines their transcriptional activation, through the increase in the DNA binding or the interaction with co-activators (Molkentin et al, 1996 A; Lu et al, 2000).

Casein kinase II (CKII) was the first kinase associated to MEF2 phosphorylation (Molkentin et al, 1996 A). It phosphorylates MEF2C on Ser59 inside the MEF2 domain and as a consequence it increases the DNA binding properties of MEF2C (Molkentin et al, 1996 A). CKII is also involved in the phosphorylation of serine 289 of MEF2A (Cox et al, 2003), but this site is also under the control of p38 (Cox et al, 2003).

In cultured cells, MEF2s are activated after serum stimulation (Kato et al, 1997). This activation is largely due to the ERK5-mediated phosphorylation and activation of MEF2s TADs. In particular ERK5 phosphorylates Ser 387 of MEF2C and activates MEF2 transcriptional programme in CHO, COS-7, NIH-3T3 and C2C12 cells (Kato et al, 1997). As a consequence, MEF2C transcribes the early genes among which *c-jun* (Kato et al, 1997). Also MEF2D is phosphorylated by ERK5 on the corresponding residue of MEF2C (Ser 430), but in this case this event does not determine the transcriptional activation of the TF, at least in HeLa cells (Yang et al, 1998). The activation of

c-jun promoter was further investigated in the following years and the activation of MEF2 by serum was attributed to multiple phosphorylation events mediated mainly by ERK5, p38α and γ (Marinissen et al, 1999). In 293 cells MEF2A and MEF2C are phosphorylated by ERK5 and p38α and to a lesser extent by p38γ. In contrast, only p38α and p38γ phosphorylate MEF2D, and none of these kinases phosphorylates MEF2B. ERK5 increases (∼20-fold) MEF2A and MEF2C (∼10-fold) activity (Marinissen et al, 1999). p38α also stimulated both MEF2A and MEF2C but to a lesser extent than ERK5, while p38γ activates only MEF2A. p38δ, MAPK, and JNK did not activate any of the MEF2 constructs and MEF2B and MEF2D were not activated by any of these kinases (Marinissien et al, 1999). In contrast to these findings, in 2000 Kato and colleagues demonstrated that ERK5 phosphorylates and activates all MEF2 members except for MEF2B (MEF2A at Thr-312, Thr-319, and Ser-355; MEF2C at Ser 387 and MEF2D at Ser 180 and 430) in CHO, COS-7, NIH-3T3 and C2C12 cells. The apparent contradiction was explained in the same year thanks to the experiments conducted by Winoto group (Kasler et al, 2000). They demonstrated that ERK5 has a functional transactivation domain and that for ERK5 mediated activation of MEF2D the kinase activity of ERK5 is dispensable (Kasler et al, 2000). Moreover, working in immature T-cells, they demonstrated that MEF2D activation exerted by ERK5 determines the transcription of *Nur77* gene. *In vivo* the effect of ERK5 requires the pre-activation of MEF2 exerted by calcium influx, which displaces Cabin1 (Kasler et al, 2000) and class IIa HDACs (McKinsey et al, 2001) from the N-terminus of MEF2s. This region is also the binding domain for ERK5 (1-92). Winoto group worked for a couple of years on the ERK5-MEF2 axis and a couple of years later they identified the ERK5 signature, by comparing the gene expression profile in ERK5 -/- fibroblasts with that in ERK5 and ERK5 (1-570) reconstituted fibroblasts (Sohn et al, 2005). Partially disavowing the results obtained four years before, they demonstrated that the signature between cells expressing full-length ERK5 and the mutant deleted in the transactivation domain (1-570) is extremely similar. Among the ERK5-regulated genes, they focused on KLF family and they demonstrate that the transcription of those genes depends on the activation of MEF2; as KLF genes control the maturation of T-cells they proposed for the first time a role of MEF2s as regulators of T-cell maturation and survival (Sohn et al, 2005).

The activation of MEF2C induced by ERK5 in PC-12 cortical neurons has a pro-survival effect. In response to the treatment with H_20_2, c-Src activates ERK5, which counteracts ischemic cellular damage also through the activation of MEF2C (Suzaki et al, 2002). The pro-survival effects attributed to ERK5 in embryonic neurons were confirmed in the following years. In particular, ERK5 mediates the development of the central nervous system also through the activation of MEF2A and C in response to neurotrophins, and in particular to BDNF (Liu et al, 2003). The activation of MEF2-signature has pro-survival effects in immature cortical neurons (Suzaki et al, 2002; Liu et al, 2003), especially through the transcription of the gene *NT-3* (Shalizi et al, 2003). The activation of MEF2 by ERK5 has prosurvival roles also in breast cancer cells that overexpress *Erbb2* (Borgess et al, 2007). In this context MEF2 inhibits TRAIL-induced cell death (Borgess et al, 2007). On the opposite, the activation of MEF2 by ERK5 has pro-apoptotic effects in neuroblastoma primary cells over-expressing the neurotrophin-3 receptor, even though the mechanism of this process is still obscure (Sturla et al, 2005). ERK5 activates MEF2C also in mast cells (Wei et al, 2003) and in endothelial cells (Hayashi et al, 2004).

MEF2 is also phosphorylated and activated by p38. The first demonstration was obtained in monocytes activated with LPS (Han et al, 1997). The activation with LPS determines the phosphorylation of MEF2C by p38 but not by Erk or JNK (Han et al, 1997). As a consequence, MEF2C transcribes *c-jun*, replenishing *c-jun* levels after LPS-induced *c-jun* consumption (Han et al, 1997). p38 phosphorylates MEF2A on residues Thr312, Thr319, Ser453 and Ser479 and MEF2C on residues Thr293, Thr300 and Ser387 (Zhao et al, 1999). These

phosphorylations determine the activation of the TAD; in the case of MEF2A the activation is maximal when MEF2A is in complex with MEF2D (Zhao et al, 1999). The activation of MEF2C exerted by p38 is necessary to achieve the muscle differentiation driven by MyoD (Zetser et al, 1999) and is sufficient to restore muscle differentiation in de-differentiated rhabdomyosarcoma cells (Puri et al, 2000). Moreover, the phosphorylation of MEF2A by p38 is a pre-requisite for its association with Smad2 and Smad4 and the differentiation of C2C12 cells driven by TGFb (Quinn et al, 2001). p38 hyper-activation and class IIa HDACs inactivation are implicated in the pathogenesis of Becker syndrome, a recessive nondystrophic myotonia caused by mutations in the chloride channel 1 gene (CLCN1) (Wu and Olson, 2002). The osmotic shock in these myotonic myoblasts induces the activation of p38, which, concomitantly with class IIa HDACs inactivation, determines the over-activation of MEF2C. MEF2C activates the transcription of a pool of oxidative and hypertrophic genes, determining the fiber-type switch characteristic of the Becker myotonia (Wu and Olson, 2002). Also in healthy skeletal muscles, p38/MEF2 axis is involved in the transformation of fast into slow fiber type. In particular, p38 phosphorylates MEF2C and this promotes the recruitment on fast fiber myosin heavy chain IId/x (MyHCIId/x) promoter of CBP/p300 (Meissner et al, 2007).

p38-mediated phosphorylation of MEF2A/C is also required for hypertrophic chondrocyte differentiation (Stanton et al, 2004). This stress-related kinase seems to act in this context as a sensor of matrix and membrane stiffness (Stanton et al, 2004). p38 regulates MEF2-related hypertrophic responses also in skeletal muscles (Munoz et al, 2009) and in cardiomyocites (Dionyssiou et al, 2013).

The activation of MEF2C via p38 is also required for the activation of HSC (hepatic satellite cells) and their expansion and proliferation (Wang et al, 2004), as examined more in details in the following paragraphs.

p38 kinase is a well-known driver of inflammatory responses (Arthur and Ley, 2013). Not surprisingly, MEF2 activation via the p38-dependent pathway mediates vascular inflammation and macrophages infiltration in vascular smooth muscle cells (Suzuki et al, 2004).

As for ERK5, also the p38-mediated activation of MEF2C in cortical neuron has pro-survival effects (Okamoto et al, 2000). Neurotrophins activate MEF2A and promote neuronal survival also through the phosphorylation of serine 192 by RSK2 (p90 ribosomal S6 kinase 2), a kinase downstream to ERK1/2 (Wang et al, 2007). On the contrary, p38 activation and MEF2A/C phosphorylation correlates with the induction of apoptosis in some ovarian carcinoma cells after the treatment with some chemotherapic agents (Holmes et al, 2003).

p38 is also involved in MEF2C phosphorylation and activation after BCR stimulation of B-cells (Khiem et al, 2008), probably together with calcineurin mediated de-phosphorylation of Cdk5 sites (Wilker et al, 2008).

Up to 2007, MEF2D was not considered a substrate of p38 MAPK. Instead, in 2007 MEF2D phosphorylation on Thr308 and 315 by p38 was demonstrated. This phosphorylation modulates the recruitment of the Ash2L coactivator complexes to muscle genes; as a consequence the methyltransferase Ash2L enhances the H3K4 methylation on the promoters of muscle genes during muscle differentiation (Rampalli et al, 2007).

As discussed, the phosphorylation of multiple sites in MEF2s determines their activation. However, there are some important exceptions where phosphorylation inhibits rather than activating MEF2s.

In particular, MEF2A/C/D were identified as substrates of the CDK-related kinase CDK5, which phosphorylates a conserved serine in the three MEF2 proteins (Ser 404, 396, 444 respectively in MEF2A, C, D) (Gong et al, 2003). The phosphorylation of these residues determines the inhibition of the transcriptional activity of MEF2 proteins, mainly because this phosphorylation acts as a priming event for PIASx that promotes the SUMOylation of MEF2A/C/D respectively on lysine K403/K391/K439 (Gregoire et al, 2006; Shalizi et al, 2006; Shalizi et al, 2007). The CDK5-SUMO axis is working both in neurons and in muscles and possibly in all post-

mitotic tissues (Gregoire et al, 2006; Shalizi et al, 2007). In hippocampal neurons, MEF2 was identified as a negative regulator of synapsis formation (Shalizi et al, 2007). In particular in this context the activity of MEF2s is regulated through a complicated but extremely elegant mechanism, which depends on the abundance of calcium (Shalizi et al, 2007). In particular, Shalizi and colleagues discovered that in response to increased neuronal activity, there is a rise in calcium in hippocampal neurons. Calcium activates the calcium/calmodulin-regulated phosphatase calcineurin, which dephosphorylates and activates MEF2A, preventing the SUMOylation of lysine 403. Once activated, MEF2A promotes the transcription of a pool of genes, including *arc, synGAP* and *Nur77* that restrict synapse number; the acetylation of lysine 403 further fixes MEF2A activation, making necessary a HDAC in order to remove the acetyl moiety and allow the SUMOylation and the repression of MEF2 activity (Shalizi et al, 2007). The antagonism between CDK5 and calcineurin seems to be a rather global phenomenon that involves both muscles and neurons (Gregoire et al, 2006, Shalizi et al, 2007). Curiously the phosphatase activity of calcineurin is active against the phosphorylation events promoted by CDK5, but not by p38 and ERK5 (Kasler et al, 2000; Wu et al, 2001; Xu et al, 2002).

Calcium plays a key role in regulating MEF2 activity. In particular CAMK (Calcium-Calmodulin Kinase) I and IV phosphorylate class IIa HDACs and promote their cytoplasmic re-localization (Lu et al, 2000), thus promoting myogenesis (McKinsey et al, 2000) and cardiac hypertrophy (Passier et al, 2000). The dissociation of class IIa HDACs from MEF2 could be also the mechanism responsible for the activation of the fraction of *GLUT4* transcribed by MEF2 (Smith et al, 2007). In this way, the calcium-mediated activation of MEF2 could regulate metabolism and could increase the rate of glycolysis, especially in red fibers. CAMKIV phosphorylates and inhibits also Prohibitin 2, a recently discovered repressor of MEF2 in muscles (Sun et al, 2011).

During glutamate-induced excitotoxicity and oxidative stress caused by H_20_2, CDK5-mediated phosphorylation is required in neurons for the cleavage of MEF2A/D but not of MEF2C by Caspase-3 and this event increases the apoptotic rates of these cells (Tang et al 2005).

The refractoriness of MEF2C to CDK5-mediated phosphorylation after exposure to glutamate is very strange. Indeed MEF2A, C and D were already described as substrates of Caspase-3 (MEF2A, C) and Caspase-7 (MEF2A, C, D) during excitotoxicity (Okamoto et al, 2002). The N-terminus products of these cleavages act as dominant-negative forms that abrogate MEF2 signaling and increase the apoptotic rates (Okamoto et al, 2002).

Putting together Okamoto and Tang papers, it is conceivable that CDK5-mediated phosphorylation of MEF2 TFs is sufficient for increasing the cleavage by caspases, but it is not necessary. The phosphorylation signal seems to be necessary for the cleavage of MEF2A and MEF2D also in apoptotic cerebellar granule neurons, and also in this case the caspase-mediated cleavage generates dominant-negative forms of MEF2 (Li et al, 2001).

The withdrawal of cerebellar granule neurons from neuronal activity determines the nuclear accumulation of GSK3β (Linseman et al, 2003; Wang X et al, 2009). In this way GSK3β interacts with MEF2D and phosphorylates Thr145, Ser149 and Ser153 in TAD1 thus inhibiting MEF2D transcriptional activity and abrogating its pro-survival effects (Wang X et al, 2009). However, the question remains of how the GSK3B is active only on MEF2D and not on other MEF2s. Recently, a model was proposed to explain this apparent paradox. In 2013, Dionyssiou and colleagues demonstrate that GSK3β inhibits myogenesis and cardiomyocyte hypertrophy (Dionyssiou et al, 2013); on the contrary, GSK3β inhibition stimulates myogenesis and MEF2 activity. Contrary to what was previously seen by Wang, they demonstrated that GSK3β directly phosphorylate MEF2s only at a very low level, but prevents its phosphorylation by p38 (Dionyssiou et al, 2013).

Another second messenger involved in MEF2 regulation in cAMP (Du et al, 2008). An increase in cAMP levels represses MEF2 activities through a double mechanism: on one hand cAMP activates PKA, which binds and

phosphorylates MEF2D on serines 121 and 190 (Du et al, 2008); on the other hand PKA phosphorylates and induces the activation of HDAC4, probably through a proteolytic (Backs et al, 2011) nuclear import (Du et al, 2008). The result of this double hit is a strong repression of MEF2 transcriptional activities, which physiologically determines a block in myogenesis (Du et al, 2008). This pathway seems to work also in lymphocytes where it negatively regulates the production of IL-10 (Liopeta et al, 2009) and in hippocampal neurons, where MEF2D phosphorylation decreases the transcription of *KLF6* and promotes apoptosis (Salma and McDermott, 2012).

PKA mediated inhibition of MEF2 is therefore a strong stimulus that could overcome the LKB1 and SIK2/3 mediated nuclear extrusion of class IIa HDACs and MEF2 de-repression, keeping off the activity of MEF2 (Walkinshaw et al, 2013).

PKA is reported to act in a completely different way in cortical and cerebellar neurons under depolarization. In this case PKA has a pro-survival effect because it phosphorylates MEF2C on threonine 20 and enhances its DNA binding capability (Wang et al, 2005).

These differences could be explained or with the different nature of the pro-apoptotic stimuli (KCl in Wang paper and increase in cAMP in Salma paper) or with the different effects of PKA stimulation on MEF2C (Wang et al, 2005) and MEF2D (Salma and McDermott, 2012).

Among MEF2 family members, MEF2C and MEF2D undergo tissue-specific splicing to generate a muscle-specific isoform (isoform α2) (Martin et al, 1994; Sebastian et al, 2013; Zhang et al, 2014). In fact, in the case of MEF2D, the alternative incorporation of the mutually exclusive third (α1) or fourth (α2) exon results in the generation of an mRNA encoding Mef2Dα1 or Mef2Dα2 isoforms; while the α1 is ubiquitously expressed, α2 isoform is muscle-specific and in particular becomes to be expressed only during advanced muscle differentiation and is absent in proliferating myoblasts (Sebastian et al, 2013). This alternative exon usage is not ineffective, as the usage of the fourth exon in place of the third in Mef2Dα2 isoform removes the Ser119 residue targeted by PKA in the Mef2Dα1 isoform and makes Mef2Dα2 resistant to PKA-mediated phosphorylation and inhibition (Sebastian et al, 2013). Moreover, while the favorite partner of the α1 isoform is HDAC9, α2 isoform interacts preferentially with the co-activator Ash2L thus providing the majority of MEF2D transcriptional activity (Sebastian et al, 2013).

In the case of MEF2C, the α2 isoform that is required for efficient differentiation of skeletal muscle cells was identified as highly down-regulated in rhabdomyosarcoma (RMS) cells, where the α1 isoform is instead the most expressed (Zhang et al, 2014). As a matter of fact, in RMS cells MEF2 transcriptional activity is highly down-regulated because between the two isoforms the α1 is more bounded by class IIa HDACs (Zhang et al, 2014). It is not clear if the loss of the PKA site in the α2 isoform releases MEF2C from class IIa HDACs repression as reported for MEF2D, but what is extremely evident in the research article is that the re-expression of the hyper-active α2 MEF2C isoform inhibits the proliferation and anchorage independent growth of RMS cells (Zhang et al, 2014). Zhang and colleagues hypothesize that SRP3, a kinase down-regulated in RMS cells, regulates the alterative splicing of the α1/α2 exon of MEF2C by activating the splicing factor ASF (Zhang et al, 2014).

Homeodomain-interacting protein kinase 2 (HIPK2), a proline-directed kinase well-known for its regulative roles in many apoptotic processes (Doxakis et al, 2004), has been recently identified as a regulator of myogenesis, mainly through its capability to repress the activity of MEF2C (de La Vega et al, 2013). In particular, it has been reported that in proliferating undifferentiated myoblasts HIPK2 is capable to associate with the MEF2C–HDAC4 complex, thus promoting the phosphorylation of the fraction of MEF2C bounded to

HDAC4 (de La Vega et al, 2013); the phosphorylation of MEF2C induced by HDAC4 was investigated for many years, but none was able to identify the kinase responsible. In 2006, Xiang-Jiao Yang group discovered that HDAC4 stimulates the phosphorylation of MEF2D on Serine 444 by CDK5 and the subsequent SUMOylation (Gregoire et al, 2006). They also demonstrated that probably other sites become phosphorylated after HDAC4 binding and that these phosphorylation events could be abrogated by LiCl (Gregoire et al, 2006). De La Vega and colleagues reported that the kinase responsible for this phosphorylation could be HIPK2 (de La Vega et al, 2013), even though HIPK3 but not HIPK2 is sensitive to LiCl (Puca et al, 2008). As a consequence of this phosphorylation event, MEF2C loses the capability to interact with CBP/p300 and becomes deacetylated by HDAC3 and repressed (de La Vega et al, 2013). Therefore HIPK2, through MEF2 phosphorylation, blocks premature differentiation; at the appropriate time during differentiation HIPK2 is degraded through a still unknown caspase-dependent mechanism and allows the entering into differentiation (de La Vega et al, 2013). Unfortunately, in this paper it was not identified the residue of MEF2C that is phosphorylated by HIPK2 (de La Vega et al, 2013).

Recently, MEF2D has been described as a novel target of ATM kinase (Chan et al, 2014). In particular, the exposure of cerebellar granule cells to stressors that induce DSBs causes the ATM-mediated phosphorylation of MEF2D on residues 259, 275, 294, 314 and the consequent increase in the transcriptional activity (Chan et al, 2014). As reported by multiple groups and as discussed in details in this paragraph, MEF2 plays a pro-survival role in neurons exposed to stressors. Chan and colleagues discovered a new mechanism capable of activating MEF2 and that is more specific for double strand DNA breaks. These findings, together with the demonstration that HDAC4 is extruded in the cytoplasm through an ATM-dependent mechanism (Li et al, 2012), candidate the MEF2-class IIa HDACs axis to the role of DSBs repair mechanism and Oncogene-Induced Senescence regulators, an hypothesis further discussed in details.

MEF2A	MEF2C	MEF2D	Kinase	Effect
Ser59, 289	Ser59		CK2	Activation, enhanced DNA binding
Ser255	Ser240		Erk5, Cdk5, GSK3β	Degradation caspase-mediated
		Thr145 Ser149 Ser153	GSK3β	TAD inhibition
Thr312, Thr319, Ser355	Thr387	Ser180 Ser430	Erk5	TAD activation
Ser192			RSK2	TAD activation
Ser98 Ser192 Thr312*vivo* Thr319*vivo* Ser453*vitro* Ser479*vitro*	Ser183 Thr293 Thr300 Ser387	Thr308 Thr315	P38	TAD activation
Ser403	Ser396	Ser444	Cdk5	Degradation caspase-mediated?
Ser403	Ser396	Ser444	Cdk5	TAD inhibition: priming phosphorylation prior to SUMOylation.
		Ser119 Ser190	PKA	TAD inhibition and binding to class IIa HDACs

	Ser20		PKA	Activation, enhanced DNA binding
		Thr259 murine Ser275 murine Ser294 murine Ser314 murine	ATM	TAD activation
?	?	?	HIPK2?	Inhibition for induced-deacetylation
Ser98,108		Ser98,106	MTORC1?	In MEF TSC2-/- phosphorylated by a kinase Rapamycin sensitive

Table 1. Scheme of the most relevant kinases involved in MEF2 phosphorylation and the residues targeted

Another important PTM capable of modulating the activity of MEF2s is the SUMOylation. All MEF2s are SUMOylated through the conjugation of SUMO-2 and SUMO-3 (Gregoire and Yang, 2005). MEFs are sumoylated within the C-terminal transcriptional activation domain inside the KXEXXSP motif (K395 in MEF2A, K324 in MEF2B, K391 in MEF2C, K439 in MEF2D), and their SUMOylation inhibits their transcriptional capability (Gregoire and Yang, 2005). MEF2s are subjected to a phosphorylation-dependent acetylation-SUMOylation switch: MEF2 phosphorylation by a kinase, in general CDK5, induces the SUMOylation of a lysine that is in general acetylated by the MEF2 co-activator CBP/p300; the removal of the acetyl-moiety which is required for the conjugation of the SUMO-group requires deacetylation by HDAC3 (Shalizi et al, 2007).
It is reported that HDAC4 potentiates the SUMOylation of MEF2s and for this activity the C-terminus HDAC domain is dispensable (Gregoire and Yang, 2005). Curiously, also HDAC4 is SUMOylated on K559 and its SUMOylation decreases the rate of MEF2 SUMOylation (Gregoire and Yang, 2005). This data is in contrast to what seen by Kirsh and colleagues that reported for the first time the SUMOylation of HDAC4 by RanBP2 and the consequent increase in the repressive capability of HDAC4 on MEF2 (Kirsh et al, 2002). For many years HDAC4 was considered as the SUMO E3 ligase of MEF2 and the co-aggregation spots of MEF2-HDAC4 complexes identified in the nucleus were considered SUMOylation-centers (Kirsh et al, 2002; Kang et al, 2006).
Now it is known that the SUMO E3 ligase of MEF2 is PIASx (Shalizi et al, 2007). The SUMO group is removed mainly by SENP2 (Lu et al, 2013). HDAC4 enhances MEF2 SUMOylation through the activation of the SUMO E2 conjugating enzyme Ubc9 (Zhao et al, 2005) and the nuclear dots of MEF2-HDAC4 are not SUMOylation centers (Gregoire and Yang, 2005), but could be aggresomes.

As anticipated, MEF2 TFs are also acetylated by CBP/p300, which enhances their DNA binding and transcriptional activities (Ma et al, 2005). Class I HDACs, and in particular HDAC3, are able to remove the acetyl-moiety and repress MEF2s (Gregoire et al, 2007). It has been recently suggested that MEF2 is itself able to recruit CBP/p300 and promote the acetylation of histones surrounding the promoter of the regulated genes (Zheng et al, 2013), but further data are required in support of this hypothesis.

Recently, it has been demonstrated that MEF2D is subjected to methylation on lysine 267 by the methyltransferase G9a (Choi J et al, 2014). In myoblasts, this methylation event takes place before differentiation and determines the inactivation of MEF2D, because of decreased binding to DNA (Choi J et al, 2014). This modification is reversible thanks to the activity of the demethylase LSD1, which removes the mono- and di-methyl groups as soon as myoblasts differentiate. As both the methylated residues and the region of MEF2D involved in the interaction with G9a (aa 1-130) are conserved among all the MEF2 members, Choi and colleagues hypothesize that this PTM could affect the transcriptional activity of all the members and also in different tissues (Choi J et al, 2014).

c) regulation of MEF2s transcription and modulation of the stability of the messengers (RNAi)

The existence of multiple pathways that converge on MEF2s makes the study of their regulation extremely complex. During myogenesis, cardiomyogenesis or lymphocytes differentiation, several TFs have been identified as inducers of MEF2s expression.

The principal activators of MEF2 transcription are:

- Twist and Myogenin during somatic myogenesis (Potthoff and Olson, 2007);
- GATA and NKX2-5 during cardiomyogenesis (Potthoff and Olson, 2007);
- Endothelin-1 during neural crest development (Potthoff and Olson, 2007);
- ETS family of TFs during vasculogenesis (Potthoff and Olso, 2007);
- PU.1 and NKX2-5 during lymphoid differentiation (Cantè-Barret et al, 2013).

The strong tissue dependency of MEF2 mRNA transcription regulation prevents the defining of a unique scheme of induction. To date, the stronger and universal inducer of MEF2 transcription is MEF2 itself (Ramachandran et al, 2008). While the genomic structure of the coding region of MEF2s is quite conserved among the members, the 5'UTR, that is more than 60 kb long, is extremely divergent (Ramachandran et al, 2008). Studying the structure of MEF2A gene, Ramachandran and colleagues identified seven non coding exons in between the transcription start-site and the ATG leader (Figure 6). A plethora of isoforms could be generated from the splicing of these non-coding exons; however the functional role of these variants is not known yet (Ramachandran et al, 2008). Ramachandran and colleagues identified upstream to the first two non-coding exons of MEF2A two alternative TATA-less promoters (p1 and p2); both of them could be bound by MEF2A itself which enhances the transcription rate of the mRNA, and makes transcription sensitive to the same regulators that affect the transcriptional activity of MEF2s, such as p38 and ERK5 (Ramachandran et al, 2008). Promoter 1 also includes a functional canonical nuclear respiratory factor 1 (NRF1) element at –53 relative to TSS p1 and potential NRF2 (–273 and –307), NF-AT (–250), and E box elements (–398 and –782) within 800 bp of TSS p1. Promoter 2 has multiple GC boxes, including three within 80 bp of TSS p2; but for both the promoters further investigations are required in order to confirm these predictions (Ramachandran et al, 2008).

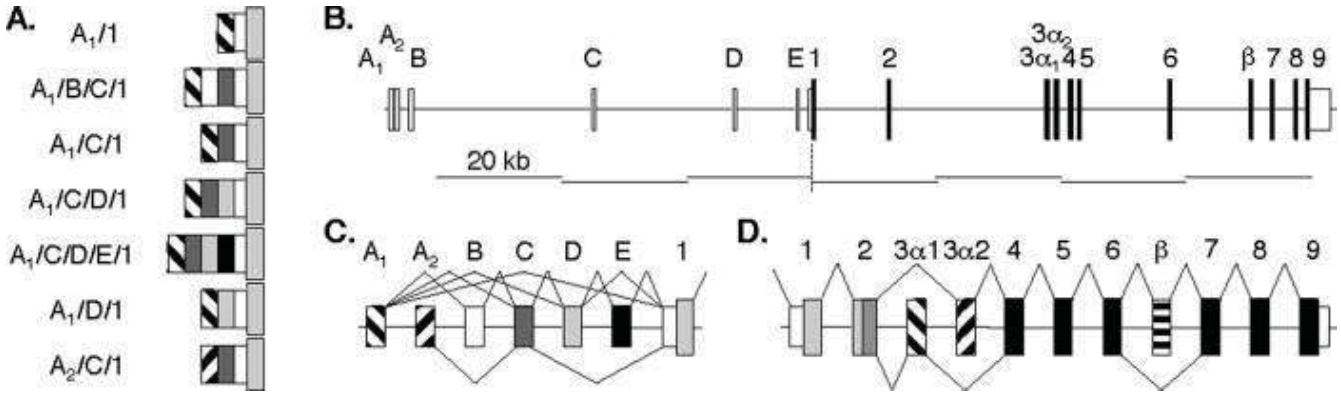


Figure 9 The complex structure of MEF2A gene. A: the different combinations after the splicing between the first seven non-coding exons of MEF2A. **B.** The complete structure of the 150 Kb of MEF2A gene. With letters are indicated the non-coding exons, with numbers the coding ones. **C.** Scheme of the splicing events among the non-coding exons**. D.** Scheme of the slicing events among the coding exons of MEF2A (Adapted from Ramachandran et al, 2008).

In the coding region of MEF2A/C/D there are nine common exons. There are two alternative third, exons 3α1 and 3α2, that are mutually exclusive and that are present in each gene (Zhu et al, 2005). As anticipated in the

previous section, the α1 isoform is ubiquitous and can complex with class IIa HDACs, while the α2 isoform is expressed only in fully-differentiated myocytes and is refractory to the binding by class IIa HDACs (Zhu et al, 2005; Sebastian et al, 2013). The vertebrate MEF2 genes could have an additional exon (β) between exons 6 and 7, which is variably included in mRNAs. Finally, a γ domain fused to the last coding exon could be excluded uniquely in MEF2C by the splicing to a cryptic acceptor site in exon 9 (Zhu et al, 2005). Therefore there are 4 isoforms of MEF2A and MEF2D (α1γ; α2γ; α1βγ; α2βγ) and 8 isoforms of MEF2C (α1; α2; α1γ; α2γ; α1β; α2β; α1βγ; α2βγ) (Zhu et al, 2005). The epta/octapeptide β domains of MEF2A (aa 287-294)/C (aa 271-278)/D (aa 286-292) are rich in acidic residues (Asp and Glu), and all have an amino-terminal residue with a hydroxylated side chain (Ser/Thr, S287 in MEF2A, S271 in MEF2C, T286 in MEF2D). The integration of this β domain enhances the transactivation capability of MEF2s probably through the phosphorylation of the Ser/Thr residue. At that time Zhu and colleague investigated a putative role of p38 in this phosphorylation but they demonstrated that p38 is dispensable for the activation (Zhu et al, 2005). Recently, ATM has been identified as the kinase that phosphorylates these residues even though it was not related to the splicing event (Chan et al, 2014). However, it is highly probable that the enhanced activity of the isoforms β+depends on the phosphorylation by ATM. In most tissues taken from mice, *Mef2a* and *Mef2d* β-mRNA vastly exceed that of *Mef2 a/d* β+ with the exception of brain, hearth and skeletal muscles, where the β+ isoform is by far the most expressed (Zhu et al, 2005). For *Mef2c* instead the β-γ- isoform is the most expressed also in muscles and in the brain (Zhu et al, 2005). An increase in β+ isoform could be seen during muscle differentiation and in general in cells that exit the cell-cycle, especially in the case of *Mef2a* and *Mef2d* (Zhu et al, 2005). Finally, the γ exon, which could be spliced out in MEF2C, has a repressive activity (Zhu and Gulick, 2004). Even though in all the tissues the γ- isoform of *Mef2c* is the most expressed, the retention of γ exon could offer an additional way of regulation of MEF2C activity (Zhu and Gulick, 2004). As for the β domain, also in this case the repressive activity is due to the phosphorylation of a serine, Ser396 in MEF2C. At that time it was not known, but nowadays we know that this serine is phosphorylated by CDK5 and this phosphorylation event promotes the SUMOylation and repression of MEF2C transcriptional activities (Shalizi et al, 2007).

Regarding the stability of mRNAs, a plethora of miRNA has been identified as capable of targeting MEF2s. miRNAs targeting of MEF2 is common during some stress-related responses, such as hypertrophy and neurodegeneration. It is outside the scope of this thesis discussing all the miRNAs, which regulate the stability of MEF2 proteins. Here we will briefly discuss only those impacting the MEF2/HDAC axis or that have a clear functional role.

The pro-oncogenic miRNA miR-21 negatively regulates the messenger of MEF2A in neurons and the expression of microRNA-21 correlates with the onset of dementia and neurodegeneration (Yelamanchili et al, 2010). miR-1 plays a strong anti-hypertrophic role in cardiomyocytes by repressing MEF2A, GATA and calmodulin (Ikeda et al, 2009). On the contrary, in myoblasts, miR-1 acts by targeting HDAC4 and activating MEF2-dependent signaling (Chen et al, 2006). miR-208 is induced during pulmonary hypertension and mediates the degradation of *MEF2C* mRNA (Paulin et al, 2015). Myogenesis is strongly regulated by miR-1, miR-133 and miR-206, which are necessary for the proper development of skeletal muscles and the heart. Indeed, alterations in their expression lead to serious defects in the formation of the two tissues (Zhao et al, 2005; Chen et al, 2006). miR-1 is expressed both in skeletal muscle and in the heart, while miR-206 is only present in the skeletal, but both are capable of promoting the differentiation of myoblasts to myotubes (Chan et al, 2006). Instead, miR-133 represses SRF and promotes the proliferation of myoblasts, thus inhibiting the differentiation. In murine C2C12 myoblasts therefore miR-1 and miR-206 pro-myogenic roles are antagonized by the pro-proliferative miR-133

(Chen et al, 2006; Kim et al, 2006). The pro-myogenic roles of miR-1 are linked to the repression of HDAC4; in this way MEF2 remains active and can contribute to the formation of myotubes (Chen et al, 2006; Niu et al, 2007). The balance between Mir-1 and Mir-133 allows the modulation of proliferative and differentiative stimuli during myogenesis (Townley-Tilson et al, 2010).

In an old study it was demonstrated that the activity of MEF2A is post-transcriptionally repressed by its 3'-UTR (nn 2251-2679), but the factor capable to mediate this inhibition was unknown (Black et al, 1997). In 2011 Seok and colleagues found that miR-155 is able to decrease the levels of the transcription factor MEF2A by targeting the 3'-UTR of MEF2A mRNA at the level of nucleotides 2534-2557. They have also shown that, in C2C12 cells, the overexpression of miR-155, by means of the negative regulation on MEF2A, has the ability to inhibit the differentiation of myoblasts (Seok et al, 2011). Finally, in the same study there were identified other microRNAs, which can bind the 3'-UTR of MEF2A: miR-9, miR-17-92, miR-19a and miR296. Although they are able to affect the expression of MEF2A, they are not directly involved in the modulation of the differentiation of myoblasts (Seok et al, 2011).

An additional microRNA specific for MEF2s is miR-223. The function of miR-223 was found in mice using an approach of loss of function (Johnnidis et al, 2008). The mutant mice were characterized by an increase in granulocyte compartment and this phenotype was due to the increase in *Mef2c* mRNA. The removal of MEF2C suppressed the expansion of myeloid precursors and corrected the neutrophil phenotype of mice lacking miR-223 (Johnnidis et al, 2008).

Finally, MEF2 was recently characterized as a regulator of the process of RNA interference through its capability to promote the transcription of several miRNAs. In particular, in a mouse model of myotonic dystrophy the protein levels of cardiac MEF2C are lower than the control. The deficit in MEF2C determines a decrease in the transcription of pri-miR-1, 133, 23, 30, 99, 145. The re-expression of MEF2 is able to attenuate the pathological condition, even by the transcription of its target miRNAs (Kalsotra et al, 2014).

d) regulation of protein stability

MEF2 proteins are diffusely studied in post-mitotic tissues (neurons and muscles); in these contexts MEF2 proteins are stable with quite long half-life (Yu, 1996).

In neurons, MEF2 proteins act as pro-survival factors. In cerebellar granule neurons that undergo apoptosis by lowering extracellular potassium, or exposed to excitotoxin glutamate, MEF2A and MEF2D, but not MEF2C, are phosphorylated by CDK5 (Li et al, 2001; Tang et al, 2005). This phosphorylation event decreases the DNA binding of the full-length proteins, increases the cell-death with a concomitant cleavage of MEF2A and D by caspases. This cleavage generates N-terminal fragments lacking the transactivation domains, which can act as dominant-negative forms that further decrease the survival rate (Li et al, 2001). The activation of CDK5 seems to follow GSK3β activation, which accumulates in the nucleus of cerebellar granule neurons exposed to low potassium, and phosphorylates and inhibits MEF2D (Wang X et al, 2009), thus abrogating its pro-survival effects (Linseman et al, 2003; Wang X et al, 2009). The generation of MEF2 N-terminal fragments after induction of apoptosis in neuronal cells is confirmed in cerebrocortical neurons exposed to excitotoxic (N-methyl-D-aspartate, NMDA) insults (Okamoto et al, 2002). In this case it was found that also MEF2C is cleaved by caspases. Authors reported all the cleavage sites for each MEF2 (MEF2A: caspase 3, 6, 7, 8 on D176, caspase 3, 7 on D215, caspase 3, 7 on D466; MEF2C: caspase 3, 7 on D422; MEF2D: caspase 7 on D288, 291). The pharmacological inhibition of caspases with zVAD was sufficient to abrogate the cleavage (Okamoto et al, 2002).

The pro-survival role of MEF2 proteins is confirmed by the cleavage of HDAC4 induced by caspase-3, which generates a N-terminus fragment that acts as a strong repressor of MEF2s (Paroni et al, 2004).
The pro-survival effects of MEF2s in neurons are also linked to their capability to regulate the plasticity of the brain (Chen et al, 2012). In particular, MEF2s may act as master regulators of developmental metaplasticity though a very simple mechanism. Upon certain stimuli, MEF2s become cleaved by caspases, thus decreasing the survival rate and causing the disappearance of some synapsis; on the contrary, others stimuli do not impact of MEF2 stability and maintain the pre-existing synapsis (Chen et al, 2012).
These reported pro-survival roles of MEF2s are subverted in some context in which MEF2s exhibit pro-apoptotic roles, mainly due to the transcription of *Nur77*. In double-positive CD4(+)CD8(+) thymocytes HDAC7 is exported in the cytoplasm and releases MEF2s that can transcribe *Nur77* causing apoptosis (Youn et al, 1999; Dequiedt et al, 2003). MEF2s induce also a caspase-indipendent cell-death in macrophages when apoptosis is inhibited with zVAD. In particular in this case in normal conditions the half-life of MEF2 proteins is relatively short (about 5 hours); the treatment with the inhibitor of caspases increases this half-life and the transcriptional activity of MEF2s. As a consequence, MEF2 proteins transcribe *Nur77*, which is responsible for the caspase-indipendent cell-death of these cells (Kim et al, 2003).
By studying the pro-survival effects of MEF2 in cerebellar granule neurons (CGNs), it was demonstrated for the first time that MEF2s undergo Ubiquitin-Proteasome Dependent degradation (UPDd) (Butts et al, 2005). Butts and colleagues cultivated the CGNs in a control medium that allows their survival in culture by exposing them to serum and depolarizing concentrations (25 mM) of extracellular potassium, then they removed the serum and they lowered the extracellular potassium from 25 mM to 5 mM causing the intrinsic apoptotic cell-death of CGNs (Butts et al, 2005). The acute treatment with the proteasome inhibitor MG-132 blocked caspase activation induced by removal of depolarizing medium. At least in part this effect was due to the inhibition of the hyperphosphorylation, ubiquitylation and degradation of MEF2D (Butts et al, 2005). In the same year, Tang and colleagues, working with the same cellular model, demonstrated that the inhibition of the proteasome increased MEF2D level also in the absence of toxicity, therefore demonstrating for the first time that the proteasome pathway is also involved in the normal turnover of MEF2D (Tang et al, 2005). In 2010, Magli and colleagues demonstrated for the first time a degradation-dependent regulation of MEF2s in proliferating undifferentiated myoblasts (Magli et al, 2010). In particular, they demonstrated that in un-differentiated C2C7 MEF2C could be found in complex with the prolyl cis/trans-isomerase Pin1. For the interaction, phosphorylation of MEF2C on four residues (Ser 98, Ser 110, Ser 240, Ser 388) is required. The catalytic activity of Pin1 results in the decrease of MEF2C stability and delays the differentiation of myoblasts (Magli et al, 2010). It is still unknown if this mechanism of regulation is active only in proliferating myoblasts or also in differentiated myocytes and if it is a general mechanism active also in other contexts such as neurons or it is cell-type specific.
Finally, it was reported that in the midbrain dopaminergic progenitor cell line SN4741 MEF2D is degraded through a process of chaperone-mediated autophagy (Yang et al, 2009). In particular, Hsc70 interacts with the cytoplasmic fraction of MEF2D (aa 1-86) and determines the degradation of MEF2D in conditions of starvation (Yang et al, 2009). The results obtained by Yang and colleagues are surprising because they focused on the cytoplasmic fraction of MEF2D, a transcription factor mainly nuclear. They also demonstrated that the autophagy-mediated degradation of the cytoplasmic MEF2D is necessary for maintaining the nuclear pro-survival function of MEF2D, and any interference with this degradation process is followed by the accumulation

of an inactive cytoplasmic fraction of MEF2D (Yang et al, 2009). Further data are required to sustain this new cytoplasmic function and regulation of MEF2D.

10. Main functional roles

It is universally accepted that the four vertebrate MEF2 genes display distinct, but overlapping, temporal and spatial expression patterns, with highest expression in striated muscles and brain (Edmondson et al, 1994; Lyons et al, 1995; Potthoff and Olson, 2007). This affirmation lies on the basis of two papers by Olson lab, in which they analyzed the patterns of expression of the transcripts of *MEF2A*, *MEF2C* and *MEF2D* by in situ hybridization of sections of post-implantation mouse embryos beginning at day 7.5 p.c. (post coitum) (Edmondson et al, 1994; Lyons et al, 1995).

During early embryogenesis, MEF2s mRNA are confined predominantly to myogenic lineages (Edmondson et al, 1994). In both the ancient cardiac and skeletal muscles MEF2C is the first MEF2 factor to be expressed, appearing initially in mesodermal precursors, which give rise to the hearth starting from day 7.5 p.c and in the somite myotome starting from day 9.5 p.c. In the primitive hearth MEF2C transcripts rise up to day 11.5 p.c. when they begin to decline. Transcripts for MEF2A and MEF2D were firstly detected at high levels in the heart at day 8.5 p.c., one day after MEF2C and they begin to decline contemporary to MEF2C.

In somites, the first bHLH factor to be expressed is Myf5 at day 8 p.c., followed by Myogenin at day 8.5 p.c., MRF4 at day 9.0 p.c , MEF2C at day 9.5 p.c. and MyoD at day 10.5 p.c. MEF2C expression rises initially in rostral somites and progresses caudally between days 9.0 and 11.5 p.c., in parallel with somite maturation. MEF2A transcripts were detected at day 9.5 p.c. and initially lag behind those of MEF2C but by day 10.5 p.c. MEF2A becomes to be expressed in myotomes more caudal than was MEF2C. MEF2A expression was also detected at low levels in the lateral mesoderm and in neural crest cells migrating away from the neural tube beginning at day 9.5 p.c. (Edmondson et al, 1994). MEF2D expression was also detectable in myotomes at days 9.5 p.c. and rises up to day 11.5, when *MEF2D* mRNAs appear in a wide range of cell types throughout the embryo. In developing smooth muscles MEF2C rises just before MEF2A and MEF2D in between days 9.0 p.c. and 10 p.c. Then the expression of the three main MEF2s diversifies, with MEF2A becoming the more expressed in the ancient blood vessels forming around the neural tube (Edmondson et al, 1994).

Regarding other districts, MEF2A and MEF2D are the MEF2s mostly expressed in developing bones, while MEF2C is the most expressed in developing gut. At day 11.5 p.c., MEF2C and MEF2A were expressed at low levels and MEF2D at higher levels in cells that will contribute to the smooth muscle of the gut.

The brain is the district in which the expression of the MEF2s diversifies more (Lyons et al, 1995). *MEF2C* mRNAs are initially detected in the CNS at day 11.5 p.c. in the telencephalic area, the region of the embryonic brain where neurons begin to differentiate. Soon after, also MEF2D and MEF2A are expressed. At day 13.5 p.c., MEF2C is expressed in the frontal cortex and in the olfactory bulb, while MEF2A mRNAs are detected predominantly in the developing thalamus. MEF2D mRNAs are less focused and are distributed uniformly throughout the neural tube (Lyons et al, 1995). In the following days, MEF2C and MEF2D becomes to be highly expressed in frontal cortex, hippocampus, amygdala, midbrain, while MEF2B rises only in the frontal cortex and MEF2A, while more pan-expressed than MEF2B, increases predominantly in the thalamus. In the later stages the four MEF2s become more overlapping and widely expressed.

In the development of CNS it is extremely evident that the pattern of expression of the various MEF2 is spatially and temporally distinct especially during embryogenesis and early in the differentiation and then becomes more homogeneous and overlapping in adult tissues. Furthermore, the level of expression of the

various MEF2s correlates with the degree of differentiation, demonstrating that the onset of MEF2 gene expression coincides with withdrawal from the cell-cycle and initiation of differentiation (Lyons et al, 1995). The correlation of expression patterns of the MEF2 genes with neuron differentiation is particularly striking in the cerebellum (Lyons et al, 1995). In this context MEF2C levels raise firstly at day 11 p.c. in precursors of Purkinje cells, concomitantly with their final mitotic divisions. An exception to this rule is MEF2D that begins to be expressed firstly at day 14.5 p.c. in the actively proliferating external granule cell layer of the cerebellum and then raises also in the inner not proliferating granule cell layer, suggesting an important role for this gene in both dividing and postmitotic neurons (Lyons et al, 1995).

The described patterns of MEF2s expression during embryogenesis suggest that MEF2s play fundamental roles in regulating many differentiative pathways in the embryo. Several evidences supports the fact that MEF2 proteins are ubiquitously expressed in well-differentiated adult tissues (Potthoff and Olson, 2007), where they both contribute to maintain differentiation and to regulate some stess-related adaptive responses (Potthoff and Olson, 2007).

Molecular pathways that normally ensure proper embryogenesis and tissue maintenance in post-embryonic life are subverted in cancer (Hanahan and Weinberg, 2011). This consideration implies an involvement of MEF2 and the axis MEF2/class IIa HDACs in carcinogenetic processes. Here I will discuss briefly the well-known roles of MEF2s in regulating both embryogenesis and adaptative responses in adult tissues, than I will analyze the complementary and accessory roles of class IIa HDACs in these processes and finally I will focus on the subversion of the physiological regulation of these pathways and the involvement of MEF2s and MEF2/HDAC axis in oncogenesis.

11. MEF2 as a regulator of differentiation programs

The functional redundancy among the four MEF2 made quite complicated the study of MEF2 in vertebrates. The existence of a single MEF2 allele in Drosophila has simplified the study of the biology of this gene in this model system. The pivotal papers are from the lab of Olson that could be legitimately considered the father of both MEF2s and class IIa HDACs and from the lab of Nguyen. The first study reporting a role of dMef2 in regulating myogenesis dates back 1995 and was from Nguyen lab (Bour et al, 1995). In this study it was demonstrated that dMef2 gene is expressed early in mesoderm and it is necessary for the complete muscle and hearth differentiation (Bour et al, 1995). In fact in dMef2 null flies there is a complete block in myoblast fusion and in the formation of the six pairs of flight muscles. Mef2d mutant flies display also several defects in cardiac cells of the dorsal vessel which are deprived of the contractile proteins, which are markers of successful differentiation (Bour et al, 1995).
During somatic muscle development, the expression of MEF2s is regulated and potentiated by three main factors: a) the bHLH transcription factor Twist that directly regulates Mef2 expression in adult somatic muscle precursor cells by binding a 175-bp enhancer located 2245 bp upstream of the TSS; b) Lame duck, a Zn-finger protein of the Gli superfamily, that induces the expression of dMef2 by binding to an enhancer different from the one bound by Twist (Duan et al, 2001); c) the Smad4 homolog Medea transcription factor, that binds a 460-bp dpp-responsive regulatory module in dMef2 promoter (Nguyen and Xu, 1998). Temporarily, dMef2 gene expression is firstly induced in mesodermal precursors mainly through the 280-bp Twist-dependent enhancer; this expression is then reinforced by Lame duck and finally, the dorsal mesoderm-restricted dMef2 expression is mediated by Medea (Nguyen and Xu, 1998; Duan et al, 2001). Finally, later in the differentiation,

dMef2 sustains its own transcription (Cripps et al, 2004). The expression of dMef2 in the cardiac lineage is controlled through the binding to a cardiac specific enhancer by the homeodomain protein Tinman and the GATA TFs (Cripps and Olson, 1998, Figure 7).

The activity of dMef2 genes during Drosophila development was dissected by Furlong group through a combinatory approach of DNA microarray and ChIP experiments (Sandmann et al, 2006). dMef2 was found to bind 1015 genomic regions in three consecutive temporal steps. This consecutive binding is not regulated through the differential expression of dMef2, as dMEF2 is present at high levels early in development but binds certain loci only at a later stage (Sandmann et al, 2006). In particular dMef2 binds the first group of enhancers at 4–6 hr of development, after which dMef2 remained bound through the three subsequent developmental time points. The second group, representing 21% of the enhancers, is bound by dMef2 at 4–6 hr of development, but it is not bound at later developmental time points. The third group, containing 32% of the enhancers, is only bound by dMef2 late in development (Sandmann et al, 2006). This study represents a milestone in the field of MEF2 for three new elements:

1. Mef2 binding to the genome should be regulated by other partners, such as Twist, which could redirect Mef2 in other genomic loci
2. Mef2 itself redirects other partners along the genome
3. Mef2 is a central regulator of myogenesis, because it does not regulate only the terminal stages of myogenesis, as previously supposed, but it is active soon after its induction and also regulates the enhancers of muscle identity genes. Then at later stages of myogenesis, MEF2 functions become more evident as it regulates a plethora of genes involved in muscle attachment, neuromuscular junction (NMJ) formation, ion transport, channel activity, metabolism and contractility (Figure 7).

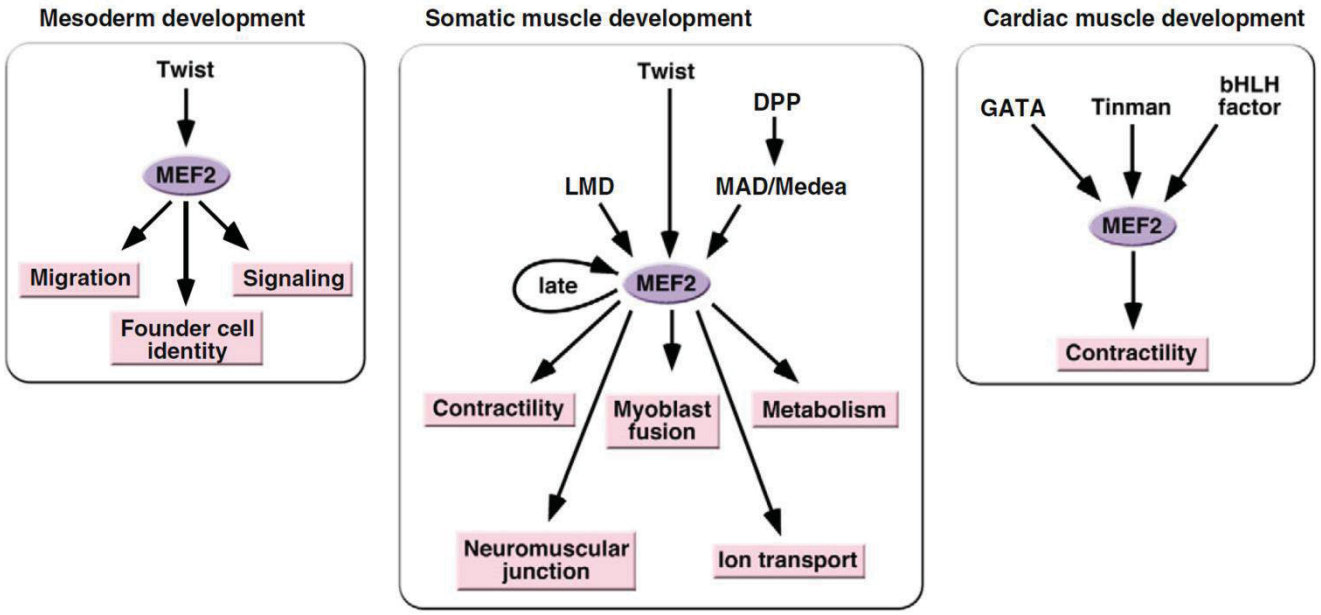


Figure 10 Different ways of regulation of dMef2 expression in somatic and cardiac muscle development in Drosophila. Mef2 activates a transcriptional programme fundamental for most of the muscular functions of these two tissues (Sandman et al, 2006). (Adapted from Potthoff and Olson, 2007).

12. A lesson from the study of the knock-outs.

As anticipated, the study of MEF2 functions in vertebrates and in particular in mammals is extremely complicated by partial redundancy among the four MEF2 proteins. For these purposes the knock-out has not ever been the most important tool in order to investigate the role of MEF2 proteins. As for much of the literature of MEF2 TFs and HDACs, the first experiments with MEF2 knock-out mice are form Olson lab. The homozygous knock-outs of MEF2A and MEF2C evidenced the key-roles of the TFs in regulating the development of the hearth and the process of cardiac myogenesis (Lin et al, 1997; Naya et al, 2002) and of endochondral bone ossification (Arnold et al, 2007). The phenotypes of these knock-outs are exacerbated by lethal phenotypes obtained by tapping these embryogenetic processes whose completion is crucial to obtain vital mice and obscure other key roles of MEF2s, as for example the regulation of skeletal muscles differentiation and of the lymphoid lineage. In fact, MEF2C null mice die by embryonic day 9.5 (E9.5) from abnormal cardiovascular development (Lin et al, 1997), whereas MEF2A null mice die perinatally from a spectrum of heart defects (Naya et al, 2002).

Mice MEF2A -/-, obtained by replacing the second coding exon of murine MEF2A with a Neo cassete, display an increased incidence of mortality soon after the birth, between post-natal day 2 and 10 (Naya et al, 2002). At a first examination the mutated mice did not presented particular phenotypic changes. The biopsy of the mice revealed severe cardiac cyto-architectural defects with ventricular chamber dilation; the mutant mice that survived to adulthood exhibit a reduced number of cardiac mitochondria and a reduced activity of cytochrome c oxidase (COX) activity indicative of a defective electron transport complex IV (Naya et al, 2002). In surviving knocked-out mice, some well-known MEF2 target genes, such as *Nur77* and α-skeletal actin, are repressed as expected, while others, such as atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), which are markers of cardiac hypertrophy and heart failure, are up-regulated in MEF2A mutant hearts. This up-regulation is due to the compensatory activation in the hearth of MEF2D (Naya et al, 2002). The phenotype of MEF2A knock-out mice reveals specific not redundant roles for MEF2A in maintaining appropriate mitochondrial content and cyto-architectural integrity in the post-natal hearts. However, it also evidences that this tool could be used only for highlighting some specific functions of the various MEF2 that could not be compensated in any way by other MEF2 (Naya et al, 2002). Moreover, the compensatory activation of MEF2D in surviving mice rises two questions: how much of the described phenotype depends on MEF2A deficiency and how much on MEF2D activation? Is this compensatory over-activation of MEF2D an extreme attempt to survive and compensate the defects of MEF2A mutants or it is always present after MEF2A deletion?

To date a MEF2B knock-out mouse has not been generated yet. Historically, MEF2B is the less studied among the MEF2 members because of two properties:

a) in respect to the other MEF2 it brings a mutation in the MADS domain, where an aspartic acid that is fundamental for the binding to DNA is substituted by a glutamine (E14Q). As a matter of fact MEF2B has a capacity to bind the DNA *in vitro* that is half of the gain of function MEF2B Q14E (Molkentin et al, 1996 B)

b) MEF2B evolves faster than the other three MEF2 proteins. The phylogenetic and evolutionist explanation of this peculiarity is that “it is reasonable that the functional constraint on MEF2B is lower than on the other three MEF2 genes. This could be why few mutant MEF2B phenotypes have ever been reported” (Wu et al, 2014).

However, Della Favera group has recently reported an oncogenic function for MEF2B in Diffuse-Large B-Cell Lymphoma (Ying et al, 2013) and they are going to describe the phenotype of the conditional knock-out mice in B-cell lineage.

Interestingly, compensatory behavior for MEF2B has been reported after the depletion of MEF2C. The complete knock-out of MEF2C causes a significant up-regulation of MEF2B (Lin et al, 1997). Moreover, in the hearth of wild-type mice MEF2B is alternatively spliced in the ventricle in respect to the atria with the retention of an intron in between the exon 5 and the exon 6. This alternative splicing is lost in mice conditionally deleted for MEF2C in the hearth (Vong et al, 2006), even though up to now the significance of this alternative splicing is unknown.

The phenotype of MEF2 knock-out mice is extreme, as the deletion of MEF2C causes embryonic lethality at day E9.5 (Lin et al, 1997). Biopsy of these embryos shows lethal cardiac defects such as a missing looping in the heart tube and the lack of right ventricle development and a subset of cardiac muscle genes was not expressed. The epiblast-conditional knock-out of MEF2D (MEF2D loxp Meox2-Cre) has no obvious phenotype (Kim J et al, 2008). In particular the mice are viable and without histological abnormalities in skeletal muscles, heart, liver, thymus, spleen and brain (Kim J et al, 2008) and MEF2D seems to not alter the lifespan, behavior, weight and fertility of the mice (Kim J et al, 2008). A more accurate investigation revealed that cardiac hypertrophy and fibrosis induced by pressure overload are suppressed in MEF2D –/– mice and that the forced cardiac expression of MEF2D is sufficient to cause severe cardiomyopathy and fibrosis (Kim J et al, 2008). Moreover, MEF2D has among the four MEF2 a non-redundant protective role in cerebellum (Chan et al, 2014). In fact, cerebellar granule cells from MEF2D-knock-out mice would manifest increased susceptibility to DNA damage induced with etoposide or irradiation compared with wild-type. In particular, DNA damage activates ATM that phosphorylates and activates MEF2D which promotes the expression of the prosurvival gene *Bcl-xL*. MEF2D -/- mice abolish the expression of *Bcl-xL* and increase the cell-death after DNA damage (Chan et al, 2014).

Considered the limitations arising from the study of homozygous knock-out mice phenotypes, the phenotypes of the heterozygous knock-out and of conditional single and multiple knock-outs were then analyzed, together with the adoption of new tools such as the study of the effects of an hyperactive form of MEF2 (MEF2-VP16, in which the MADS/MEF2 domains of a MEF2 are fused to the activation domain of the transcription factor VP16 of H. *symplex*) or of a super-repressor MEF2 (MEF2-Engrailed, in which the MADS/MEF2 domains of a MEF2 are fused to the repressor domain of the Engrailed co-repressor of Drosophila).

Studying the heterozygous MEF2C +/- vital mice, researcher from Olson lab noticed postnataly a lack of ossification within the sternum; moreover, MEF2C +/− MEF2D +/− mice display a more severe deficiency in sternum ossification (Arnold et al, 2007). These results were confirmed by deleting MEF2C genes specifically in proliferating chondrocytes by using a Cre transgene controlled by the *Col2a1* promoter and amplified by the simultaneous conditional deletion of MEF2C and MEF2D or by inducing the expression of a super-repressor form of MEF2C (MEF2C-Engrailed) (Arnold et al, 2007). These results demonstrate that both MEF2C and MEF2D are required for getting the chondrocyte hypertrophy and the subsequent growth plate maturation (Arnold et al, 2007). Moreover, MEF2C/MEF2D hyper-activation is sufficient to induce chondrocyte hypertrophy and endochondral bone ossification *in vivo*, because the induction of a hyper-active form of MEF2C (MEF2C-VP16) stimulates both the processes. Finally, the precocious and ectopic ossification of endochondral cartilage in mice lacking HDAC4 could be perfectly explained by the activation of MEF2C and the phenotype of MEF2-VP16 mice (Arnold et al, 2007) is perfectly compatible to that of HDAC4 knock-out mice (Vega et al, 2004).

The role of MEF2s and of the MEF2-class IIa HDACs axis in myogenesis

dMef2 mutant flies are characterized by a complete block in somatic and cardiac myogenesis (Bour et al, 1995). The role of MEF2s in regulating myogenesis in mammals is however less pronounced. MEF2 TFs alone do not

possess intrinsic myogenic activity but, in combination with myogenic basic-helix-loop-helix (bHLH) transcription factors, they amplify and potentiate the myogenic differentiation program (Potthoff and Olson, 2007). Paraphrasing, MEF2 factors are not sufficient for the differentiation of myoblasts, but are needed for getting the complete differentiation. In fact, the forced expression of MEF2A or MEF2C or MEF2D in 10T½ fibroblasts is not sufficient to induce the myogenic conversion of these cells (Molkentin et al, 1995), contrary to the over-expression of each of the four myogenic bHLH proteins, MyoD, myogenin, myf5, and MRM (Olson, 1990). However, the exogenous expression of MEF2C together with MyoD or myogenin is sufficient to dramatically increase the extent of myogenic conversion and the number of multi-nucleated myotube formed (Molkentin et al, 1995). In particular, the MEF2s are capable to interact with the bHLH proteins thanks to their DNA binding domain and in order to get a cooperative effect only one of the couple of the two TFs should have a functional transactivation domain (Molkentin et al, 1995). The dispensable role of MEF2s in driving myogenesis was demonstrated also *in vivo* by over-expressing an hyper-active form of MEF2C fused to the VP-16, which was not capable to drive a premature muscle differentiation (Potthoff et al, 2007 A). However, the expression of MEF2C-VP16 in fast-fiber-muscles is sufficient for the successful switch to slow-fiber-muscles (Potthoff et al, 2007 A).

As the first MEF2 espressed during embryogenesis in myotome is MEF2C, it was considered to be the stronger driver of skeletal muscle differentiation among the MEF2 members. In order to study the contribution of MEF2C in myogenesis, a skeletal muscle specific deletion of MEF2C was performed by inducing the knock-out using the myogenin-Cre system. In this way MEF2C was deleted starting from the embryonic day 8.5 and the knock-out resulted in 100% lethality in a C57BL/6 mixed genetic background, with pups smaller than wild-type ones and lethargic (Potthoff et al, 2007 B). No perinatal lethality was instead reported by deleting MEF2C starting from embrionic day 18.5 using a muscle creatine kinase promoter based inducible Cre (MCK-Cre) (Potthoff et al, 2007 B).

The central role of MEF2C in myogenesis was confirmed by Olson group by comparing the effects of the myogenin promoter mediated knock-out of MEF2C with that of MEF2A and MEF2D that are ineffective and completely vital (Potthoff et al, 2007).

Hystological inspections of MEF2C knock-out mice at perinatal day 1 demonstrated that the deficiency of MEF2C does not prevent the formation of complete skeletal muscles, but myofibers deteriorate after birth because of disorganized sarcomeres and incomplete M lines (Potthoff et al, 2007 B). The phenotype of these MEF2C null mice demonstrate that MEF2C is effectively not necessary for driving myogenesis *in vivo*, but it is required for the maintenance of the sarcomere integrity of the muscles (Potthoff et al, 2007 B). These results reveal a key role for MEF2C in the maintenance of sarcomere integrity and postnatal maturation of skeletal muscle (Potthoff et al, 2007 B). Moreover, MEF2C allele was the unique among MEF2 family not dispensable for the preservation of sarcomeres and it is responsible for the induction of the other MEF2s, which amplify and accelerate the process of myogenesis (Potthoff et al, 2007 B; Potthoff and Olson, 2007).

Nowadays, four stages are described during myogenesis and for each step the transcription factor absolutely needed has been identified (Knight and Kothary, 2011).

The first stage is the maintenance and the activation of the quiescent muscle satellite cells. Paired-box protein 7 (Pax7) is the TF required for the maintenance of the population of quiescent satellite cells and the Pax7 positivity Pax3 negativity distinguishes the activated satellite cells. Then, there is the proliferative step when the myogenic factor 5 (Myf5), together with Pax7, promotes the expansion of activated myoblasts. Myoblast determination protein (MyoD) is necessary for the differentiation of activated myoblast, and acts together with

myogenin and MEF2s to drive the complete differentiation and to get the myotubes fusion and the multinucleation. Finally, muscle-specific regulatory factor 4 (MRF4) is required to get hypertrophy, with the transcription factors MEF2 playing a fundamental role in the regulation of pathological cardiac hypertrophy (Knight and Kothary, 2011).

MEF2s promote myogenesis also because they induce the expression of some bHLH TFs, such as myogenin, which in turn induces MEF2C (Pothoff and Olson, 2007 B). In differentiating muscles a feed-forward circuit feeds the differentiation: MEF2C induces the expression of myogenin (Edmondson et al, 1992), which in turn induces MEF2C (Dodou et al, 2003); MEF2C induces the expression of itself and of the other MEF2s (Ramachandran et al, 2008). Moreover MEF2 promotes the expression of miR-1 that represses HDAC4 and drives myogenesis and of miR-133 that represses SRF and promotes the proliferation of myoblasts, thus inhibiting the differentiation (Chen et al, 2006). MEF2C activates also the expression of HDAC9 (Haberland et al, 2007). The existence of multiple positive and negative-feedback loops allows the perfect modulation of the process and of MEF2 activity (Haberland et al, 2007; Potthoff and Olson, 2007).

The role of MEF2 TFs in cardiomyogenesis

MEF2C null mice die because of strong defects in the cardiac looping; however cardiomyocytes coming from these knocked-out mice are still able to differentiate (Lin et al, 1997). The defective cardiac looping is the main cause of mice death. This is proved by a specific deletion of MEF2C, after the looping morphogenesis, which has no relevant negative effects on cardiac morphogenesis (Vongh et al, 2005). The dispensable role of MEF2C during murine cardiomyogenesis is due to partial redundancy and compensation among the MEF2s. As expected, the expression of a super-repressive form of MEF2C (MEF2C-Engrailed) results in embryos that lack some heart structures and that are characterized by defective cardiomyogenesis (Karamboulas et al, 2006). MEF2C in particular is necessary for the development of the anterior hearth field (AHF), which gives raise to the outflow tract (OFT) and to the right ventricle (RV) (von Both et al, 2004). On the basis that MEF2C -/- mice resembled FOXH1 -/- mice, von Both and colleagues investigated a putative cross-talk between the two TFs. They found that MEF2C is induced by FOXH1 and it is responsible for the production of the structural proteins necessary for the development of AHF (von Both et al, 2004; Potthoff and Olson, 2007).

The role of MEF2 TFs and of the MEF2-class IIa HDACs axis in endochondral bone ossification

In vertebrates there are two types of ossification: intramembranous or endochondral ossification. The former occurs through the direct conversion of mesenchymal precursors into bone, while the latter requires a cartilaginous intermediate (Hall and Miyake, 1995).

The endochondral bone ossification involves the differentiation of mesenchymal precursors to chondrocytes that proliferate and secrete matrix to give a cartilaginous intermediate. At this point the chondrocytes cease to divide and become hypertrophic (Potthoff and Olson 2007). Finally, the chondrocytes die by apoptosis, leaving holes that are filled from the vessels and osteoblasts that begin to secrete bone matrix. The chondrocyte hypertrophy is therefore the critical phase of the process and is positively regulated by MEF2C and D, which induce the expression of RUNX2, a transcription factor that positively regulates chondrocyte proliferation and hypertrophy via IHH (Indian Hedgehog Homolog)(Arnold et al, 2007; Potthoff and Olson, 2007). HDAC4, by repressing MEF2, is the main negative regulator (Arnold et el, 2007). Mice HDAC4 - / - die during embryonic life for abnormal and precocious ectopic ossification, recapitulating the phenotype of RUNX2 overexpression (Vega et al, 2004). Conversely, the overexpression of HDAC4 inhibits hypertrophy and the differentiation of

osteoblasts (Vega et al, 2004). The absolute necessity of MEF2C during endochondral ossification was demonstrated by Arnold and colleagues in 2007 when they created mice conditional knocked-out of MEF2C or over-expressing a mutant super-repressor of MEF2C, devoid of the AD domain. In both the cases they observed a complete lack of chondrocyte hypertrophy and ossification. Finally, the over-expression of a hyperactive mutant of MEF2C (MEF2C-VP16) recapitulated the phenotype given by the knock-out of HDAC4 (Arnold et al, 2007).

Differently from the dispensable role of MEF2s during muscle differentiation, MEF2s are therefore absolutely necessary and sufficient to drive chondrocyte hypertrophy (Arnold et al, 2007).

Recently, the degradation of MEF2D through autophagy in the nucleus pulposus of old rat intervertebral discs has been put in correlation with their age-related degeneration (Ye et al, 2011), suggesting not only a role of MEF2 TFs in driving chondrocyte hypertrophy but also in maintaining the integrity of the structures.

The role of MEF2s and of the MEF2-class IIa HDACs axis in vasculogenesis and differentiation of vascular smooth muscle cells

As explain further in details, MEF2s are required for a proper vascular development (Lin et al, 1998) and a correct balance between MEF2 activation and their repression by HDAC7 is require for the maintenance of vascular integrity (Chang et al, 2006). MEF2C becomes to be expressed in endothelial precursors starting from embryonic day 8.5 (De Val et al, 2004) and the conditional deletion of MEF2C in this compartment results in endothelial cells' proliferation arrest, vascular death and lethality (Hayashi et al, 2004). Going on with the differentiation, MEF2s should be repressed by HDAC7 for the correct proliferation of vascular smooth muscle cells (Zhao et al, 2012) and for the maintenance of vascular integrity (Chang et al, 2006).

The role of MEF2 TFs in neuronal development

As previously discussed, the brain is the district characterized by a clear distinction in the temporal and spatial expression of MEF2s during murine embryogenesis (Lyons et al, 1995). The differential distribution and expression among the MEF2s has the effect of stimulating the expression of different set of genes, considering that each MEF2 has a preferential binding site to chromatin (Anders et al, 1995).

Lipton group reported that a conditional knock-out of MEF2C in neuronal/stem cells starting from embryonic day 9.5, therefore early during embryogenesis, causes an abnormal distribution of neurons in the cortical plate and the formation of an immature neuronal network with fewer synapses in respect to the control (Li et al, 2008). The authors described this decreased synaptic phenotype as similar to an autism-like syndrome (Li et al, 2008).

On the contrary, in the same year Olson group reported that in their set of experiments MEF2C facilitates learning and memory by limiting the formation of excessive synapse numbers (Barbosa et al, 2008). In their experiments they knocked-out MEF2C in the brain during late embryogenesis and they obtained that the knock-out potentiates synaptic transmission in the dentate gyrus and increases the number of excitatory synapses, without affecting cell death/survival pathways. These effects could be reversed by the overexpression of the super-active mutant VP16 of MEF2C (Barbosa et al, 2008). On these bases Olson and colleagues assumed that *in vivo* MEF2 limits the excessive increase in the number of excitatory synapses (Barbosa et al, 2008).

The two papers were published back-to-back in the same number of PNAS and in the discussion Lipton was asked to justify the completely different results obtained. The proposed explanation was that "MEF2C has

pleiotropic effects on different temporal points in the neurogenic program" and that the "increased synaptic phenotype" described by Olson after MEF2C knock-out resembles more the results obtained in knock-down experiments performed on maturing cortical neurons *in vitro* (Li et al, 2008).
As a matter of fact, the results obtained by knocking-down MEF2A and MEF2C in cortical neurons by Greenberg lab are in agreement with Olson results. In fact, in Greenberg lab it was demonstrated that MEF2A and MEF2D are key negative regulators of synaptogenesis (Flavell et al, 2006). In particular, in response to any increase in neuronal activity, the intracellular levels of calcium raises and the phosphatase calcium-dependent calcineurin dephosphorylates and activates both MEF2A and MEF2D, preventing their SUMOylation respectively on lysines 403 and 439 (Flavell et al, 2006). The activation of MEF2s determines a feed-back inhibition in the process of synaptogenesis, as MEF2s promote the transcription of a pool of genes that determine the synaptic disassembly by promoting the internalization of glutamate receptors (Flavell et al, 2006). MEF2s are also negative regulators of dendritic claw differentiation and also in this case the CDK5/Calcineurin pathway regulates their activity.
Certainly, considering the temporal-dependent expression and distribution of MEF2 proteins during brain development, it is actually plausible to assume that a MEF2 could play different roles during development. Confirming this hypothesis, MEF2s play a fundamental pro-survival role in developing cortical neurons but not in mature ones (Liu et al, 2003).
In all these studies the contribution of class IIa HDACs is neglected and a putative explanation to the reported irreconcilable roles of MEF2 could be that class IIa HDACs could be competent for MEF2C binding during a certain step in the development and transform MEF2 in transcriptional repressors whereas in another later phase as transcriptional activators.

The role of MEF2s in hematopoiesis and T cell development

MEF2s are well-known regulators of hematopoiesis (Canté-Barret et al, 2013). Through a complex set of knock-out and knock-in experiments it was demonstrated by many groups that MEF2C regulates precursor cell commitment toward lymphoid development over myeloid development and toward monocyte differentiation over granulocyte differentiation within the myeloid lineage (Canté-Barret et al, 2013).
MEF2D plays fundamental roles in the regulation of positive and negative selection of immature T-cells. In particular MEF2D promotes the negative selection, that consists in the elimination of immature T-cells double positive (CD4+/CD8+) or self-reactive, through the transcription of the pro-apoptotic gene *Nur77*, which contains two MEF2-consensus sites in the promoter (Martin et al, 1994). In unstimulated T lymphocytes, MEF2D is maintained repressed through two mechanisms:
- a Cabin1 dependent mechanism, in which Cabin1 associates to the fraction of MEF2D bound to the DNA and recruits HDAC1 and HDAC2 that deacetylate the promoter of *Nur77* (Youn et al, 1999)
- a class IIa HDAC dependent mechanism, that is highly based on HDAC7, the class IIa HDAC more expressed in the thymus (Dequiedt et al, 2003). The existence of a mechanism alternative to Cabin1 to repress MEF2-dependent *Nur77* transcription was suggested by the fact that a mouse transgenic model expressing a mutant form of Cabin1 defective in MEF2 binding did not display defects in thymocytes development and apoptosis (Esau et al, 2001). TCR activation causes the PKD1 mediated phosphorylation and export in the cytoplasm of HDAC7 (Parra et al, 2005), with the consequent activation of MEF2D and *Nur77* transcription (Dequiedt et al, 2003). The de-phosphorylation of HDAC7 14-3-3 sites by PP1β phosphatase and by MYPT1 causes its nuclear import and the repression of *Nur77* (Parra et al, 2007).

The role of MEF2s in melanogenesis

Recently, MEF2C has been described as an important regulator of murine melanocytes development (Agarwal et al, 2011). In particular, the deletion of MEF2C in murine neural crest results in mice with a reduced number of melanocytes (Agarwal et al, 2011). From a molecular point of view MEF2C is induced during melanogenesis by Sox10 and acts as a down-stream amplifier of the transcriptional pathways activated by Sox10 (Agarwal et al, 2011).

The role of MEF2s in neural crest development

It is reported that MEF2C promotes the craniofacial development as the conditional knock-out mice of MEF2C in neural crest cells die because of obstruction of the upper airways and are characterized by craniofacial defects (Verzi et al, 2007; Argawal et al, 2011). MEF2 seems to be part of the Endothelin-DLX5/6 pathway (Verzi et al, 2007), but while data are convincing in Danio *rerio* (Miller et al, 2007), further data are required in mammals (Potthoff and Olson, 2007).

13. MEF2/HDAC axis' roles in well-differentiated adult tissues: evidences of regulation of hypertrophy, metabolism and synaptic plasticity

MEF2s regulates many adaptive/stress related responses in muscles, such as atrophy and regeneration responses, the exercise-induced switch from glycolytic to oxidative fibers and pathological cardiac hypertrophy.

MEF2C and MEF2D are also involved in the determination and development of the slow-oxidative muscle fibers (Potthoff et al, 2007 A). In fact, the homozygous deletions of MEF2C or MEF2D in the murine soleus (a slow-oxidative fiber type muscle) result in a reduced number of oxidative fibers, while homozygous deletions of MEF2A are ineffective (Potthoff et al, 2007 A). As anticipated, the over-expression of MEF2C-VP16 in the plantaris (a fast-fiber type rich muscle) is sufficient to increase the number of oxidative-slow type myofibers, a phenotype that is perfectly mimicked by the deletion of at least four alleles of HDAC4, HDAC5 and HDAC9 (Potthoff et al, 2007 B). Physiologically, the exercise-induced switch from glycolytic to oxidative myofibers is characterized by a decrease in the amount of class IIa HDACs protein levels in the slow-type myofibers (Potthoff et al, 2007 A). This effect is obtained through an increase in the proteasome-mediated degradation of these HDACs, which determines the activation of MEF2 transcriptional activities (Potthoff et al, 2007 A). MEF2s are therefore key-regulators of the metabolism of the muscles and are capable to increase the oxidative capacity and the mitochondrial content of the skeletal muscles (Potthoff et al, 2007 A), at least in part through the transcriptional induction of peroxisome proliferator-activated receptor–γ coactivator-1α (PGC-1α). The dependency of PGC-1α regulation from the class IIa HDACs-MEF2 axis was confirmed in another study by inducing the expression of HDAC5 in the hearth (Czubryt et al, 2003). The induction of HDAC5 causes the precocious death of male mice characterized by the loss of cardiac mitochondria and a strong down-regulation of PGC-1α (Czubryt et al, 2003). It is reported that class IIa HDACs promote muscular atrophy by repressing *Dach2* and thus activating the transcription of *Myogenin* and of its targets, the E3-ligase Atrogin-1 and MURF1 that activate the atrophy-program and the miR-206 that targets the 3'UTR of HDAC4 and HDAC5, feeding a negative feed-back (Moresi et al, 2010).
A wonderful MBC paper in 2009 by Goldman group described a complex feed-forward/feed-back circuit regulating HDAC4 activity in innervated and denervated muscles (Tang et al, 2009). This paper for many aspects synthetizes years of study on atrophy; however, some revolutionary new data have not been well received by

the scientific community, in part because some of the data seemed partially at odds with those obtained from the Olson lab, in part because the involvement of MEF2 has not been investigated. As previously described, in normal muscles MEF2 transcriptional activity is extremely active in oxidative fibers-rich muscles, such as the soleus (Potthoff et al, 2007 A). In this context, MEF2 acts as a driver of oxidative phosphorylation and class IIa HDACs, acting as MEF2 repressors, as drivers of glycolysis (Potthoff et al, 2007 A and B). This picture is completely subverted during atrophy. HDAC4 is a well-known regulator of atrophy and it acts mainly by inducing and activating *Myogenin* (Moresi et al, 2010). In denervated muscles, Tang and colleagues reported that HDAC4 acts as strong repressor of glycolysis (enolase and phosphofructokinase enzymes), probably through MEF2 repression, and a strong activator of synaptogenesis (nicotinic acetylcholine receptor and muscle-specific receptor tyrosine kinase). Therefore in this context HDAC4 acts as a switcher that redirects energy consumption in order to promote re-innervation (Tang et al, 2009). We recently confirmed these data through a DNA-microarray analysis in which we identified that the over-expression of a hyper-active form of HDAC4 is capable to significantly repress *Eno3* expression (unpublished results). Therefore it is conceivable that in a normal context, in which glycolysis is not exasperated, MEF2 is active and promotes oxidative phosphorylation through its engagement with PCG-1α promoter (Potthoff et al, 2007 A). The shift towards a glycolytic phenotype, induced by atrophy but also by oncogenes, could redirect MEF2s on other glycolytic promoters, such as enolase and phosphofructokinase promoters, which could promote glycolysis. In this view MEF2s acts as positive regulators and drivers of metabolism.
The expression of the glucose transporter *Glut4* is mainly due to the activity of the transcription factors MyoD, MEF2 and TRα1. The gene is highly expressed in oxidative muscles and less expressed in glycolytic muscles and this differential expression is mainly due to the different activity of MEF2s in the two different contexts (Moreno et al, 2003). During denervation, *Glut4* transcription is no more dependent on MEF2 (Moreno et al, 2003) and this evidence confirm that during denervation a complete resetting of MEF2 binding capability should take place, thus explaining the opposite phenotypes described for the MEF2-HDAC4 axis in regulating glycolysis in normal and denerved muscles.

MEF2s are also required for the successful muscle regeneration after an injury (Liu et al, 2014). In this case MEF2A/C/D functions are highly redundant and the single knock-outs in cardiac satellite cells do not affect the regeneration capability (Liu et al, 2014). Instead, the contemporary deletion of MEF2A, C, and D in satellite cells completely blocks the regeneration process, without affecting the proliferation capability of these cells, but affecting the transcription of a large set of coding and noncoding RNAs, that define the so called "MEF2-dependent transcriptome associated with skeletal muscle regeneration" (Liu et al, 2014).

MEF2s are reported to be hyperactive during muscle hypertrophy induced both by exogenous stimulation with IGF-1 (Musarò and Rosenthal, 1999; Bodine et a, 2001; Munoz et al, 2009) and endogenous activation of mTOR in a LKB1 -/- context (Lai et al, 2013). The first direct demonstration of the involvement of MEF2s in hypertrophy dates back 1995, when MEF2s were found to be hyperactive in a murine model of myocardial hypertrophy (Doud et al, 1995). In particular MEF2s are hyperactive both during normal and pathological cardiac hypertrophy (Kolodziejczyk et al, 1999). Over-expression of either MEF2A, MEF2C or MEF2D in transgenic mice is sufficient to induce dilated cardiomyopathy (Xu et al, 2006; Kim J et al, 2008), while the conditional hearth knock-out of MEF2D makes mice resistant to cardiac hypertrophy, fetal gene activation, and fibrosis in response to pressure overload and beta-chronic adrenergic stimulation (Kim et al, 2008). The activation of MEF2C during pathological hypertrophy is due to: the activation of p38 and ERK5 that phosphorylates and activates MEF2C (Kolodziejczyk et al, 1999; Zhao et al, 2009); the activation of CaMKI, IV

and PKD1 that phosphorylate and inactivate class IIa HDACs (Passier et al, 2000; Zhang et al, 2002 B; Fielitz et al, 2008); the increase in the transcription of MEF2s induced by FAK (Peng et al, 2009).

The loss of class IIa HDACs and the activation of p38 pathways activate MEF2s also in skeletal muscles from myotonic mice and this activation results in the transcription of oxidative and hypertrophic genes (Wu and Olson, 2002). These results were partially denied recently by Cooper group. In their work, Kalsorta and colleagues investigated the altered miRNA and mRNA expression patterns in myotonic dystrophy heart murine tissues (Kalsorta et al, 2014). They found out that many of the miRNA and mRNA repressed in myotonic mice are MEF2 targets and this dysregulation is due to a decrease in the levels of MEF2A and MEF2C (Kalsorta et al, 2014).

Recently, increasing evidences attribute to MEF2s key roles in the regulation of synaptic plasticity (Flavell et al, 2008; Akhtar et al, 2014). Curiously it has been reported that MEF2 activity is regulated by cocaine; in particular cocaine administration enhances the phosphorylation of Ser408 of MEF2A and Ser444 of MEF2D by decreasing the activity of calcineurin (Pulipparacharuvil et al, 2008). The decrease in MEF2 activity causes an increase in the density of dendritic spines in nucleus accumbens. This mechanism should be interpreted as a physiological response in order to decrease the neuronal activity and buffer the toxicity associated to cocaine abuse. In fact the forced re-expression of MEF2 results in increased sensitivity to repeated cocaine administration (Pulipparacharuvil et al, 2008) and, on the contrary, the overexpression of HDAC4 in the nucleaus accumbes reduces drug motivation (Wang et al, 2010).

Finally, I will focus here briefly on the extra-muscular metabolic functions of MEF2s. Recently, an exciting manuscript described the unique dMef2 Drosophila TF as an “In Vivo Immune-Metabolic Switcher” (Clark et al, 2013). In particular, in the fat body of healty flies, dMef2 is phosphorylated by Akt or S6K on Thr20 and promotes the expression of lipogenic and glycogenic enzymes that control anabolic processes (Clark et al, 2013). After an infection, the S6K pathway is blocked, dMef2 is dephosphorylated and associates with TBP and relocalizes on TATA-box containing promoters and promotes the expression of antimicrobial peptides (Clark et al, 2013). The involvement of MEF2s in the regulation of metabolic processes in non-muscular context is not new. It was already reported in 2011 that in neurons MEF2D activates the mitochondrial complex I, through the direct transcription of NADH dehydrogenase 6 (She et al, 2011). This gene is localized on mtDNA and what was astonishing at that time was that MEF2D was reported to be capable to localize in the mitochondria. The activation of Complex I has a protective role in neurons and Parkison disease (PD) is characterized by a decrease in the mitochondrial localization of MEF2D; authors suggested that this delocalization could contribute to PD (She et al, 2011).

14. MEF2s as regulators of proliferation and cancer

Literature offers numerous and contradictory demonstrations of both pro-oncogenic and tumor suppressor roles of MEF2s. They could be due to the capability of these TFs to regulate two processes: the differentiation and the angiogenesis.

The capability of MEF2s to stimulate the differentiation of myoblasts (McDermott et al, 1993), cardiomyocytes (Karamboulas et al, 2006), osteoblast (Arnold et al, 2007), neurons (Li et al, 2008), B-lymphocytes and monocytes (Cantè-Barret et al, 2013) points towards a tumor suppressor role of MEF2 in these districts. Although this model seems to hold true in muscles (Zhang M et al, 2013 and 2015), bones (Correa-Cerro et al, 2011) and in T-cell leukemia (Homminga et al, 2011), the oncogenic properties of MEF2B in diffuse-large B cell

lymphoma (Ying et al, 2013) and of MEF2C in liver hepatocarcinoma (Ma et al, 2014) strongly oppose against it and suggest that the situation is much more complicated than expected. Sources of complications that should be taken into account are the influences of MEF2 on cell-cycle regulation that may diverge greatly from cell lineages and the pro-angiogenic properties of MEF2, which are extremely strong in some tissues (Lin et al, 1998).

In muscles, MEF2s are involved in the differentiation process (McDermott et al, 1993). In this context, MEF2 become active as soon as myoblasts stop to proliferate (Lu et al, 2000). Myoblasts differentiation and myocytes fusion in myotubes require in fact both EGFR and JAK1-STAT1 signaling shutting down, cell-cycle arrest and entering in the quiescent post-mitotic state of G0 (Olson, 1992; Sun et al, 2007; Leroy et al, 2013). Cell-cycle arrest is a fundamental requisite of differentiating myoblasts. CDKi are required for blocking mitogenic signals and as a matter of fact mice lacking both p21 and p57 do not form myotubes because myoblast continue to proliferate and muscles arrest the development at the same step of Myogenin knock-out mice (Zhang et al, 1999). Myotubes are also characterized by Rb ipo-phosphorylation and activation that seems to be necessary both for MyoD and MEF2 nuclear localization and activation (Novitch et al, 1999).

As aforementioned MEF2s alone do not induce myogenesis, but they participate together with other myogenic regulatory factors (MRF) to the myogenic differentiation program (Molkentin et al, 1995). Also cell-cycle arrest per se is not sufficient to induce differentiation (Kitzmann and Fernandez, 2001). A fascinating hypothesis combines MEF2 and cell-cycle regulation together, trying to explain the activation of MEF2 as the consequence of cell-cycle arrest and at the opposite their repression as a matter of proliferation. This hypothesis stem from the evidence that CDK4 can interfere with the transactivation properties of MyoD, by displacing MyoD homodimers and E-12 heterodimers from the DNA, without inducing the phosphorylation of MyoD (Zhang et al, 1999). Starting from Peterson findings about MyoD and CDK4, Lazaro and colleagues demonstrated that in myoblasts CDK4-CyclinD1/3 act also as negative regulators of MEF2A/B/C/D activities. In this case CDK4 kinase activity is not dispensable for repressing MEF2. In particular CDK4 is required in order to phosphorylate once among MEF2 and GRIP-1. As a consequence of this still undefined phosphorylation event MEF2 dissociates from the co-activator GRIP-1 and its transactivation potential is strongly diminished (Lazaro et al, 2002). After ten years this hypothesis has not been verified in an optimal manner yet. Recently, Nakajima and colleagues tried to apply Peterson-Lazaro model to cardiomyocytes, but they found that the negative regulation of cardiomyocytes differentiation induced by cyclin D1 is not due to MEF2 modulation but to GATA4 destabilization (Nakajima et al, 2011). Moreover, in muscle satellite cells MEF2s seem not to be able to dramatically influence proliferation (Liu et al, 2014). In particular, Olson group studied the requirement of MEF2s in tibialis anterior (TA) muscles regeneration after an injury induced through injection of the cardiotoxin (CTX) (Liu et al, 2014). They demonstrated that the single knock-outs limited to satellite cells (Pax7-directed knock-out) of MEF2A, MEF2C and MEF2D did not have any effect in terms of muscle regeneration capability (Liu et al, 2014). Instead, the triple knock-out of the three MEF2 was sufficient to abrogate almost completely muscle regeneration (Liu et al, 2014). As the process of muscle regeneration requires both activation and expansion of satellite cells and differentiation of myogenic progenitors into myotubes, the authors investigated if MEF2s do affect one or both these processes. They found that while the triple knock-out only slightly and not significantly increased the percentage of EdU positive cells, the triple knock-out myoblasts failed to differentiate (Liu et al, 2014). This observation is in contrast with what demonstrated recently by Choi and colleagues (Choi MC et al, 2014). Using the same model of TA regeneration after CTX injection, they demonstrated that HDAC4 is required for the expansion of satellite cells after an injury (Choi MC et al, 2014). In

particular HDAC4 stimulates the expression of Pax7, of its target genes and the proliferation of satellite cells. This phenotype is restricted to injured muscles and the knock-out of HDAC4 in satellite cells activated by an injury prevents their correct expansion and differentiation (Choi MC et al, 2014). A possible explanation for the apparent contradiction of the two models may be that part of HDAC4 effects reported by Choi could be independent from MEF2. Alternatively the degree of MEF2 repression exerted by HDAC4 could be higher than MEF2 knock-out, transforming MEF2 in a super-repressor capable of influencing also satellite cells proliferation.

Similarly to what observed by Liu and colleagues, MEF2C is reported to be fundamental *in vivo* for the correct differentiation of nestin-expressing neural stem/progenitor cells (Li et al, 2008). Instead, MEF2C is dispensable for the proliferation or survival of those cells (Li et al, 2008). A big limitation of both Liu and Li studies is that in both the cases they knocked-down MEF2C in undifferentiated cells where MEF2C is almost absent; therefore they may observe a phenotype only during differentiation, when MEF2C expression increases and the comparison between the two conditions becomes significant. As a matter of fact, in the supplementary Li and colleagues state that "At E18.5 [the embryonic day in which they perform all the experiments] in cerebrocortex, MEF2 transcriptional activity is distributed in a rostral-low to caudal-high gradient and restricted to postmitotic neurons that are negative for the proliferative marker Ki67"; therefore on these basis a study of the influence of MEF2 on proliferation could not be done. Readers should bear in mind that the influence of MEF2 on proliferation could only be studied in a context where MEF2 does not regulate also differentiation, which generally involves an arrest in proliferation, or in post-mitotic not proliferating cells.

MEF2D instead significantly affects the proliferation of Rhabdomyosarcoma cells (Zhang M et al, 2013). Rhabdomyosarcomas (RMS) are rare soft-tissue sarcomas of children and adolescents. There are two main types of RMS: ERMS (Embryonal, the most frequent 60%), characterized by a loss of heterozygosity at the IGF-2 locus, and ARMS (Alveolar, 20%), characterized by PAX3/7-FOXO1A fusion (Missiaglia et al, 2012). Myocytes affected by these malignancies are locked in a proliferating undifferentiated myoblast-like status, despite the expression at physiological levels of myogenic differentiating factor such as Myf5, MYOD and myogenin, which appear not to be functional (Tapscott et al, 1993). Among the differentiation markers, both ARMS and ERMS are negative for MHC and MEF2D (Zhang M et al, 2013), MEF2C is repressed in RMS in respect to normal muscle cells (Tremblay et al, 2014), while the other MEF2 member are expressed at the same levels of the control (Zhang M et al, 2013). The loss of MEF2D expression keeps RMS myoblasts undifferentiated and the re-expression of MEF2D is sufficient to activate muscle differentiation and cell-cycle arrest through p21 up-regulation (Zhang M et al, 2013). Zhang findings were partially confirmed by Tremblay and colleagues in another model of ERMS induced by YAP1 exogenous hyperactivation (Tremblay et al, 2014). In this case YAP1 keeps cells in the very immature state of activate satellite cells, positive for Pax7 and Myf5. YAP1 represses the expression of MEF2C and MEF2D, induces the expression of two MEF2 repressors such as Twist1 and Cabin1 and redirects residual MEF2C and D away from the promoters of myogenic differentiation genes (Tremblay et al, 2014), accordingly to Olson group results (Liu et al, 2014).
One year after their publication on MEF2D, Zhang and colleagues, working on the basis of what discovered in Dilworth lab (Sebastian et al, 2013), demonstrated that RMS cells are characterized by the expression of α1 isoform in place of α2 isoform of MEF2C (Zhang et al, 2014). The former is more bounded by class IIa HDACs in respect to the latter and this fact, together with the loss of MEF2D expression, contributes to keep MEF2 signature off (Zhang et al, 2014).

Also p38 MAPK inactivation in RMS contributes in keeping off MEF2 transcriptional programme (Puri et al, 2000).
MEF2 acts therefore as negative regulators of muscle proliferation. This happens also in VSMCs (Vascular Smooth Muscle Cells) where a deletion mutant of MEF2A acts as a dominant negative and stimulates the proliferation and migration of these cells (Zhao et al, 2012).

This model in which MEF2 negatively affects proliferation by inducing cellular differentiation is subverted in hepatocytes. In particular it has been demonstrated that MEF2 induces the activation of HSC (Hepatic Stellate Cells) and stimulates their proliferation (Wang et al, 2004). Comprising 5%–8% of the resident liver stromal cells, HSCs are the principal storage site for retinoids in normal liver and they are responsible for the production of extracellular matrix and various growth factors, which regulate the proliferation and differentiation of hepatocytes (Bachem et al, 2006). In response to an injury, the normally quiescent HSCs undergo a progressive activation called transdifferentiation, becoming proliferative and contractile myofibroblasts (Bachem et al, 2006). During this activation phase, MEF2A, C and D mRNA and protein levels increase (Wang et al, 2004). This was associated with enhanced MEF2 DNA binding and transcriptional activity, due to p38 mediated activation of the TAD. MEF2s stimulate *in vitro* the expression of alpha-smooth muscle actin and the activity of collagen I promoter and increase HSC proliferation, with a decrease of the cells in G0/G1. *In vivo* an increase in MEF2 proteins correlates with fibrosis (Wang et al, 2004). Moreover the increase in HSC activation induced by MEF2s is in some way correlated with hepatocarcinoma pathogenesis (Bai et al, 2008). On the opposite it was reported that also HDAC4 is involved in the stimulation/activation of HSC cells (Mannaerts et al, 2013); this means that the class IIa HDAC-MEF2 axis is not working in these cells.

Besides their role in HSCs activation, MEF2s have also a direct proto-oncogenic activity in hepatocytes, by stimulating epithelial-mesenchymal transition (EMT), which is associated with the induction of proliferation and invasiveness. The treatment of hepatocytes with TGFb activates PI3K/AKT pathway, which induces EMT (Yu et al, 2014). As a consequence of this activation, the levels of MEF2A, C and D increase. MEF2s increase EMT response and invasiveness and induce the expression of TGFb that sustains the process, feeding a feed-forward circuit (Yu et al, 2014). In particular MEF2D, but not MEF2A, B and C, is increased in HCC samples in respect to not tumoral, and this increase is due to miR-122 down-regulation (Ma et al, 2014). In HCC cells, MEF2D binds the promoters of genes *(RPRM, GADD45A, GADD45B and CDKN1A)* involved in the negative regulation of G2/M transition and in complex with an unknown co-repressor keeps them untranscribed, thus promoting proliferation and mitosis (Ma et al, 2014). Recently, as previously described, MEF2C was found to act as a negative regulator of hepatocarcinoma cells proliferation through β-catenin cytoplasmic retention and inactivation (Bai et al, 2014); this strong anti-proliferative quality is counter balanced by the MEF2C-dependent stimulation of angiogenesis and migration. As a matter of fact, the injection of hepatocarcinoma cells overexpressing MEF2C gives rise to small slowly proliferative tumors but highly malignant because of VEGF-pathway activation (Bai et al, 2014).

The situation, as mentioned, is very complex in the immune system, considering the multitude of physiological functions that MEF2s play in this context. In particular MEF2 by promoting the differentiation of a particular immune cell type promotes also conversely the de-differentiation of another one (Cantè-Barret et al, 2013). This multifaceted activity must be taken in account in analyzing MEF2 oncogenic/tumor suppressor properties.

MEF2s, and MEF2C in particular, have key role in regulating hematopoiesis (Cantè-Barret et al, 2013). MEF2C is highly expressed in hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs) and common

lymphoid progenitors (CLPs), with the latter that is the district in which it is mostly expressed (Stehling-Sun et al, 2009 and figure 8). Then while the differentiation process goes forward its expression decreases both in the lymphoid and in the myeloid lineages (Schüler et al, 2008; Stehling-Sun et al, 2009 and figure 8). In the myeloid lineage MEF2C is more expressed in monocytes and less in granulocytes and erythrocytes (Schüler et al, 2008). In the lymphoid cels MEF2C is highly expressed in B-lymphocytes and it is almost absent in T-lymphocytes; among lymphoid organs MEF2C is highly expressed in mature follicular spleen B cells while it is much lower in marginal zone B cells (Stehling-Sun et al, 2009). The complex regulation of MEF2C levels is due to its role as regulator of myeloid and lymphoid lineage differentiation (Stehling-Sun et al, 2009). With a set of very elegant experiments it was demonstrated that the knock-out of MEF2C in hematopoietic stem cells (HSCs) determines a decrease in megakaryocytes, lymphocytes, granulocytes and monocytes numbers (Stehling-Sun et al, 2009) and an increase in granulocytes and neutrophils (Schüler et al, 2008). Its over-expression in granulocyte/monocyte–macrophage progenitors determines the differentiation of monocytes to the detriment of granulocytes (Schüler et al, 2008). MEF2C therefore acts as the arbiter of precursor cell commitment and pushes towards lymphoid development over myeloid development and toward monocyte differentiation over granulocyte differentiation within the myeloid lineage (Cantè-Barret et al, 2013). At the level of multi-potent progenitors (MPPs) MEF2C acts by facilitating the development of lymphoid over myeloid lineage (Stehling-Sun et al, 2009). The knock-out of MEF2C therefore prevents the differentiation of lymphocytes. The few cells that successfully differentiate are completely normal, suggesting that MEF2C has only a role in the early stages of differentiation and that it is essential to prevent myeloid differentiation (Stehling-Sun et al, 2009). In fact, in MPPs MEF2C -/- the stimulation with cytokines (Flt3L and IL-7) that normally favor the lymphoid development still allows the myeloid differentiation. MEF2C is induced by the transcription factor PU-1 that directs the lymphoid differentiation and the PU-1 knock-out prevents the lymphoid differentiation. As the re-expression of MEF2C in a PU-1 -/- context is sufficient to get a partial recovery of lymphoid differentiation, this means that MEF2C is downstream to PU-1 (Stehling-Sun et al, 2009). Recently it has been demonstrated that MEF2C acts mainly as a repressor of myeloid differentiation rather a promoter of lymphoid differentiation (Barneda-Zahonero et al, 2013). In fact, in B cell precursors MEF2C is bound and repressed by HDAC7; MEF2C therefore could not promote the differentiation of myeloid lineage (Barneda-Zahonero et al, 2013). This model is supported by two evidences: first, HDAC7 is expressed in B cell precursors and not in macrophages and its transcription is completely abolished during the transdifferentiation of pre-B cells into macrophages, even though the mechanism beyond this regulation is still obscure; secondly, the re-expression of HDAC7 during the transdifferentiation prevents the acquisition of macrophages markers (Barneda-Zahonero et al, 2013).

MEF2C is required for B cell proliferation and survival after stimulation of the BCR with anti-IGM, but not after the stimulation with LPS, anti-CD40, IL-4 or BAFF (Wilker et al, 2008); therefore MEF2C is required for the modulation of adaptive immune response (that depends on BCR stimulation) but is dispensable for the regulation of innate immune response (that is BCR independent) (Wilker et al, 2008; Khiem et al, 2008; Cantè-Barret et al, 2013). In particular BCR stimulation induces MEF2C activation through calcineurin mediated MEF2C dephosphorylation. The depletion of MEF2C in anti-IGM stimulated B-lymphocytes prevents their activation and proliferation, because of lack of cyclin D2 induction (Wilker et al, 2008). This evidence was confirmed independently, in the same year by Khiem and colleagues. They demonstrated that the conditional knock-out of MEF2C in B-lymphocytes tremendously affects their proliferation in response to BCR stimulation with anti-IgM but not when stimulation of Toll-like receptors with LPS was used (Khiem et al, 2008). BCR activation resulted in a 10-fold increase in MEF2 transcriptional activation, through a p38 dependent

mechanism. The over-expression of a phospho-dead mutant of MEF2C (in the p38 sites T293A, T300A, S387A) resulted in 90% reduction in proliferation after BCR stimulation respect to the control, confirming the centrality of p38 in MEF2C activation (Khiem et al, 2008).

Despite this important role of MEF2 in regulating mature B-cell activation, both MEF2C and MEF2D over-expressions do not impact on central or peripheral B cell development (Andrews et al, 2012). Andrews and colleagues found that MEF2C is less expressed in transitional B-cell in comparison to mature B-lymphocytes, while MEF2D behaves in the opposite manner (Andrews et al, 2012). The different MEF2C abundance between transitional and mature B-lymphocytes reflects their different behavior after BCR engagement. In fact while at the transitional stage a strong cross-linking BCR signal induces apoptosis, in follicular mature B-cells it induces survival and proliferation (Andrews et al, 2012). Andrews and colleagues therefore attributed to the developmentally controlled MEF2C levels the unique function of lymphocytes maturation metronomes (Andrews et al, 2012).

miR-223 null mice are characterized by a marked neutrophilia due to an increase in the total number of granulocyte progenitors (Johnnidis et al, 2008). The privileged target of miR-223 is MEF2C and its increase coming from miR-223 knock-out is the main responsible for the hyper-proliferative phenotype of granulocyte-monocyte progenitors (Johnnidis et al, 2008). In fact the genetic ablation of MEF2C suppresses progenitor expansion and corrects the neutrophilic phenotype in miR-223 null mice (Johnnidis et al, 2008). This finding is consistent with the identification of MEF2C as the mRNA strongly up-regulated during the commitment of granulocyte-monocyte progenitors into leukaemic stem cells (LSCs) induced by over-expression of MLL-AF9 chimera (Krivtsov et al, 2006). MEF2C increase is necessary but not sufficient for the maintenance of both the characteristics of stemmness and tumorigenicity of the LSCs (Krivtsov et al, 2006). This result is in contrast with what obtained by Schwieger and colleagues (Schwieger et al, 2009). In 2009 they demonstrated that MEF2C is dispensable for both establishment and maintenance of LSCs generated by MLL/ENL fusion. In particular by comparing the phenotype of the inducible conditional MEF2C knock-out MLL/ENL mouse with the MEF2C +/+ MLL/ENL mouse they demonstrated that MEF2C is not required for the induction of leukemia but shortens the latency of the disease and increases the infiltration of leukemic cells in the spleen (Schwieger et al, 2009). These two aspects are due to the fact that MEF2C silencing impacts on homing and motility of LSCs. MEF2C knock-out has instead no effects in terms of proliferation and BrdU positivity. Hence, MEF2C has a marginal role in the establishment of leukemia, but instead modulates the clinical manifestation of the disease (Schwieger et al, 2009). There are two weak points in this work: i) the dispensable role of MEF2C in LSC establishment is in contrast with the fact that the same authors identified MEF2C as a common integration site in MuLV-induced myeloid tumors of Irf8−/− mice; moreover these proviral integrations determine a 5-fold up-regulation of MEF2C transcript, but by analyzing a dataset of 285 patients with AML the authors found a strong positive and not a negative correlation between IRF8 and MEF2C (Schwieger et al, 2009); ii) in order to gain insight into the mechanism by which MEF2C may increase invasion properties of MLL/ENL transformed cells, authors performed a microarray analysis comparing MEF2C -/+ cells with cells that over-expressed an ASR hyperactive mutant. This mutant of MEF2C (VLL65ASR) is known for its inability to bind class IIa HDACs and therefore should acts as a hyperactive (Molkentin et al, 1996 A); however, the same authors that firstly generated this mutant described that surprisingly it is transcriptionally inactive (Molkentin et al, 1996 A; Lu et al, 2000). Therefore the genes identified by Schwieger as significantly up-regulated by MEF2C ASR over-expression, belonging to chemokine-receptors and matrix metalloproteases, should be a large underestimation.

MEF2C was also found induced in an AML model coming from HSC transduced with NUP98-HOXA10 (NA10) fusion protein (Palmqvist et al, 2007). Palmqvist and colleagues also identified in human samples of AML a significantly and tight correlation between the expression levels of MEF2C and PRNP genes, another gene induced by NA10 fusion. Moreover both of them showed a high expression in patients with unfavorable cytogenetics. However, their expression did not correlate with HOXA genes in human leukemia. This observation suggests that their induction may be restricted to NUP98-Hox fusions and that they are not regulated by HOXA5, HOXA7, HOXA9 or MEIS1 in human bone marrow cells (Palmqvist et al, 2007).

MEF2C is reported to represent the central oncogene for immature T-acute lymphoblastic leukemia (Homminga et al, 2011). In particular MEF2C is found rearranged and overexpressed in an immature cluster of T-ALLs (Homminga et al, 2011). The rationale of this induction is that MEF2C is required for keeping T-lymphocytes undifferentiated and immature. As expected MEF2C silencing in the T-cell acute lymphoblastic leukemia cell line LOUCY induces their differentiation and decreases proliferation. Some of the pro-proliferative effects of MEF2C could be explained by TGFb pathway modulation; however Homminga and colleagues have some doubts about, also in consideration that MEF2 induces both some positive (such as TGFBR1) and some negative regulators of TGFb (such as SMURF2 and SMAD7). In order to further prove the transforming potential of MEF2C, Homminga and colleagues selected fibroblasts, as classical models of transformation. They demonstrated that MEF2C is able to transform murine NIH-3T3 fibroblasts in cooperation with RAS and BJ-EHT cells in association with either RAS or MYC (Homminga et al, 2011). These results are surprising because in their experimental conditions RAS is not sufficient to transform NIH-3T3 cells; secondly, because in BJ-EHT human fibroblasts MEF2C has the capability to escape oncogene-induced senescence by its own, making the effects of MEF2 comparable to the sum of those of E1A and MDM2.

As aforementioned, the model that describes MEF2s as a tumor suppressors in the districts in which it induces differentiation and as an oncogene where favors the maintenance of an undifferentiated state works well for the reported pro-oncogenic roles of MEF2C in myeloid and T-cell leukemia (Krivtsov et al, 2006; Palmqvist et al, 2007; Schwieger et al, 2009; Homminga et al, 2011). The model provides that MEF2s should not have an oncogenic role in B lymphocytes. Actually, despite MEF2C is highly expressed in common lymphoid progenitors and in the B-cell compartment, to date no MEF2C aberrations have been described in leukemic B precursors, with the exception of the DAZAP1/MEF2 translocation (Prima et al, 2005). In fact, In TS-2 cells, a cellular model of B-ALL, the variant t(1;19)(q23;p13.3) was identified, which encodes for reciprocal in-frame chimeric protein DAZAP1/MEF2D and MEF2D/DAZAP1 (Prima et al, 2005). The two chimeras display similar oncogenic properties in NIH-3T3 cells and the co-expression of both together boots the effects of the single chimeras (Prima et al, 2007). DAZAP1 is a RNA binding protein involved in mRNA splicing regulation, characterized by two N-terminus RRM (RNA-recognition motif) and a low complexity C-terminal domain (CTD) proline-rich (Choudhury et al, 2014). In the DAZAP1/MEF2D chimera the two N-terminus RRM domains of DAZAP1 are fused with the TAD2 of MEF2D (Prima et al, 2005), while in MEF2D/DAZAP1 the DNA binding and dimerization domains and the TAD1 of MEF2D are fused to the CTD of DAZAP1. Curiously, while DAZAP1/MEF2D loses the capability to bind the MEF2 consensus-site on DNA, the fusion of DAZAP1 to the C-terminus of MEF2D increases the transcriptional capabilities of MEF2D (Prima et al, 2005). Despite the opposite effects in terms of MEF2 activation, the two chimeras display similar oncogenic properties in NIH-3T3 cells (Prima et al, 2007).

The proposed model is completely subverted in diffuse large B-cell lymphomas (DLBCLs), mantle lymphomas and follicular lymphomas where MEF2C and MEF2B mutations are frequent (Lohr et al, 2012; Zhang J et al,

2013; Ying et al, 2013; Bea et al, 2013) and MEF2B behaves as an oncogene (Ying et al, 2013). In particular DLBCLs are the tumors in which the rate of MEF2B somatic mutations is higher (7,5%). The 81% of MEF2B reported mutations affect the N-terminal region of MEF2B, while the others affect the TADs or are missense/frameshift mutations that generate truncated proteins (Ying et al, 2013). MEF2C is also mutated in DLBCL (Morin et al, 2011; Lohr et al, 2012), but with a lower frequency (2%) and more importantly MEF2B is the MEF2 expressed at higher levels in germinal center B-cells (Ying et al, 2013). Among the identified MEF2B point mutations, only one (K4E) affected MEF2B transcriptional potential, 5 mutations have no effects (L38I, N81Y, G105E, R171X, R207Q), other 5 increase MEF2 transcriptional activity (L54P, Y69H, E77K, S78R, D83V) (Ying et al, 2013). All the substitutions that increase MEF2 transcriptional activity and also the mutation N81Y abolish the interaction of MEF2 with the repressor CABIN1. Two identified mutations determine the premature truncation of the protein (R171X and Y201X) and other three (G242fs, P256fs and L269fs) determine a frameshift. Both these kind of mutations conserve the function of the protein but alter the frame or create a trunk to the carboxy-terminal that prevent the phosphorylation of MEF2B at serine 324 by PKA. Importantly, this phosphorylation mediates the binding of Ubc9 and the SUMOylation and inhibition of MEF2B, and therefore all these mutations have gain of function effects (Ying et al, 2013).

In discussing about MEF2 pro-oncogenic and tumor suppressor properties, it cannot be left out a quick analysis of the effects of mitogen stimulation on MEF2. MEF2s belong to SRF family of transcription factors and with the members of the family share the property of being activated by serum stimulation (Han and Prywes, 1995; Kato et al, 1997). Serum activates MEF2C in two ways: by enhancing the binding of MEF2D to DNA (Han and Prywes, 1995) and by promoting the activity of the activation domain of MEF2A and C by direct phosphorylation induced by kinases, principally MAPK (Kato et al, 1997). Whatever the mechanism, the activation reaches a peak within an hour and is exhausted within four hours (Han and Prywes, 1995; Suzuki et al, 1995). As a consequence of this activation MEF2s promote the transcription of the immediate early response genes including *c-jun* (Han and Prywes, 1995; Suzuki et al, 1995; Kato et al, 1997). Curiously, in NIH-3T3 fibroblasts MEF2D is reported to be incompetent for *c-jun* transcription (Han and Prywes, 1995). It is still unknown and not investigated how much of *c-jun* transcription depends on MEF2s and whether MEF2s can influence re-entering the cell-cycle after starvation. In contrast to this model, MEF2C has been seen to be down-regulated at mRNA level by ERK/MAPK pathway during G1/S transition in NIH-3T3 cells (Yamamoto et al, 2006). The forced expression of MEF2C in these cells, during late G1 phase blocks S phase entry (Yamamoto et al, 2006).

Finally, at least a part of the oncogenic properties of MEF2s could be due to their ability to regulate the processes of vasculogenesis (Lin et al, 1998) and angiogenesis (Shang et al, 2013). The establishment of new blood vessels is a complex process that requires the aggregation of angioblasts to form the primitive vascular plexus, their proliferation followed by differentiation and finally their migration and coalescence to form the primitive vessel (Drake, 2003). MEF2 seems to have a double role in this process, because it promotes the first phase of aggregation-expansion-differentiation of angioblasts (Lin et al, 1998), but its transcriptional activity should be repressed in order to preserve vascular integrity (Chang et al, 2006; Shang et al, 2013). In particular the knock-out of MEF2C results in severe vascular abnormalities and lethality in homozygous mutants before embryonic day 9.5 (Lin et al, 1998). These vascular abnormalities are not due to a failure in endothelial cells differentiation but to their inability to organize into a vascular plexus. This phenotype is also due to the failure in VSMCs differentiation. In fact these fully-differentiated cells are required for organizing the plexus in mature blood vessels (Lin et al, 1998). As the phenotype of these MEF2C -/- mice resembles that of VEGF -/- animals, Lin and colleagues hypothesized an involvement of MEF2s in VEGF transcription (Lin et al, 1998). This

involvement in VEGF transcription was demonstrated in the hearth (Bi et al, 1998), where the severe endocardial defects in MEF2C knocked-out mice are largely due to a reduction in angiopoietin 1 and VEGF mRNA production by the myocardium (Bi et al, 1998). Although the direct dependency of VEGF transcription from MEF2 is still obscure, accumulating evidences point towards an involvement of VEGF in the activation of MEF2 (Maiti et al, 2008; Xu et al, 2011). Activation of MEF2 could be due to a VEGF-induced phosphorylation (Maiti et al, 2008) or its de novo transcription (Rivera et al, 2011). Once activated MEF2 could itself stimulate VEGF cascade (Maiti et al, 2008; Xu et al, 2011).

The phenotype of HDAC7 -/- mice results in embryonic lethality due to severe defects in blood vessels; these are caused by a loss in cell-cell adhesion between endothelial cells (Chang et al, 2006).
This phenotype is recapitulated in human endothelial cells silenced for HDAC7 and the loss of cell adhesion is due to the up-regulation of the endoproteinase MMP10 (Chang et al, 2006).
MMP10 is a gene transcribed by MEF2 and therefore, as aforementioned, while MEF2 activation is necessary in the initial steps of vasculogenesis, its repression mediated by class IIa HDACs is necessary for the maintenance of endothelial integrity (Chang et al, 2006; Potthoff and Olson, 2007).
Recently, a TGFβ dependent and HDAC7 independent mechanism for achieving MEF2 repression in endothelial cells has been also described (Shang et al, 2013). In this case TGFβ activates TAK1, which phosphorylates and activates HIPK1 and HIPK2. HIPK1 and 2 in turn repress MEF2 probably through direct phosphorylation and the effect of this repression is the decrease in the transcription of MMP10 (Shang et al, 2013).

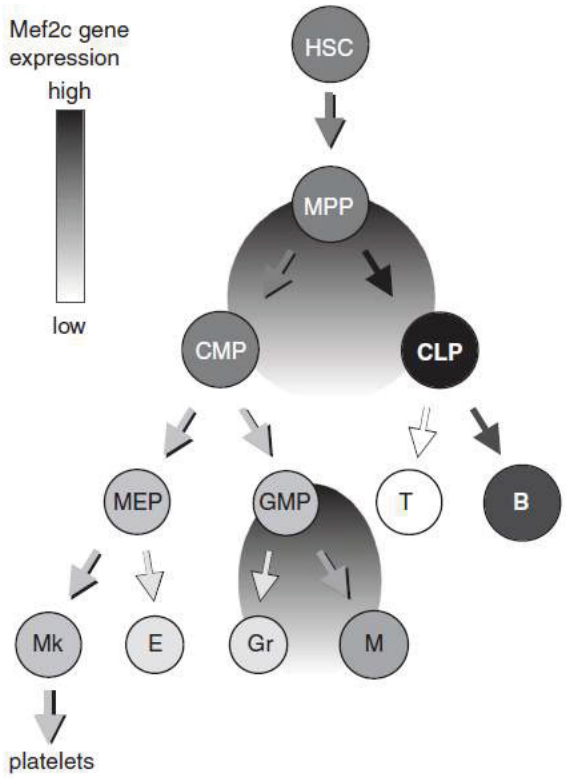


Figure 11 MEF2C is expressed in a not homogenous way in the different hematopoietic lineages and at different stages of blood cells differentiation B: B-lymphocyte; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; E, erythrocyte; Gr, granulocyte; GMP, granulocyte–monocyte/macrophage progenitor; HSC, hematopoietic stem cell; M, monocyte; MEP: megakaryocyte–erythrocyte progenitor; MPP: multipotent progenitor; Mk: megakaryocyte; T, T lymphocyte (Figure adapted from Cantè-Barret et al, 2013).

MEF2 and NOTCH1

A separate chapter deserves the cross talk between Notch and MEF2. Increasing evidences show that the two signaling pathways may act sometimes agonistically and sometimes antagonistically. However, this paragraph was not included in the chapter devoted to "MEF2 and cancer" because, although Notch is increasingly counted among oncogenes (Lobry et al, 2014), to date there is still no evidence about the existence of a cross-talk between Notch and MEF2 even in tumors. Notch1 and Notch3 are well-known inhibitors of muscle differentiation (Shen et al, 2006). This inhibitory function is achieved through the induction of the anti-myogenic factor and MyoD repressor HES1. However, at least part of the anti-myogenic properties of Notch1 is HES1 independent (Shawber et al, 1996). In particular, the intracellular domain of Notch1 (ICN1) is capable of ablating MEF2C activities, thus repressing terminal myoblasts differentiation (Wilson-Rawls et al, 1999). Notch1 interacts with aminoacids 105-115 of MEF2C, a region adjacent to the MEF2 domain and that is not conserved among the other members of MEF2 family. As a consequence of this interaction, MEF2C loses its capability to bind the DNA (Wilson-Rawls, 1999). Moreover, Notch1 is also involved in the displacement of the co-activator MAML1 (Mastermind-like transcriptional Coactivator) from MEF2, probably through competitive binding (Shen et al, 2006). This competition is double: MAML1 interacts with aminoacids 87-177 of MEF2C, in a region that is also bound by Notch1, but also MEF2C and Notch1 compete for the binding to MAML1. Therefore Notch1 signaling abrogates both the transcription of MEF2C induced by MAML1 recruitment on MEF2C promoter and its phosphorylation and activation induced by the formation of MEF2C-MAML1-p300 complex (Shen et al, 2006). B lymphocytes are physiological models of high competition between Notch and MEF2 pathways (Demarest et al, 2008). In particular Notch inhibits B-cell development by inducing MAPK-mediated ubiquitylation and degradation of E2A transcription factors and JNKs, that are both required for normal B-cell development (Nie et al, 2008). Notch instead does not negatively alter T-cell maturation, where MAPK and JNK signals are weak (Nie et al, 2008). On the opposite, Notch3 stimulates T-cell development without perturbing T-cell maturation (Van de Walle et al, 2013). MEF2 transcription factors act exactly at the opposite by driving B-cell maturation over T-lymphocytes differentiation (Cantè-Barrett et al, 2014).

The linearity of this competition model is broken in Drosophila, where the intracellular domain of Notch (ICN) and MEF2C cooperate for the binding to the promoter of the transcription factor *Egr*. As a result they activate both JNK and Matrix Metalloproteinase and they induce proliferation (Pallavi et al, 2012). MEF2 and Notch 1/3 protein levels correlate in breast cancers specimens providing another level of complexity (Pallavi et al, 2012). To get scenario even more complex, recently it has been published that MEF2C is capable of repressing Notch signature in another model of terminal muscle differentiation in Drosophila (Caine et al, 2014). MEF2 exerts its repression on NOTCH by inducing the transcription of its ligand (Delta) at very high levels to engage a cis-inhibition mechanism based on Michaelis-Menten kinetics (Caine et al, 2014).

As we have discussed, our knowledge about the MEF2-NOTCH axis are still in their infancy and it will be very interesting to see if the antagonistic model will be confirmed within the tumors.

AIMS

As explained in details in the introduction, the involvement of the MEF2/class IIa HDACs in oncogenesis is still debated. In particular, in the last two years several reports pointed towards a pro-oncogenic role of MEF2 TFs in some contexts, such as immunological cancers (Cantè-Barret et al, 2013) and hepatocarcinomas (Ma et al, 2014). Many of these manuscripts claim tumor-promoting roles for these TFs on the basis of their up-regulation or of the tumor-suppressor effect of their knock-down.

The concept of MEF2 TFs as transcriptional repressors when bound to class IIa HDACs is not new, but it is difficult to assimilate (Shalizi et al, 2007). The strong repression exerted by class IIa HDACs allows MEF2 TFs to bind the genome for switching off the transcription of some MEF2 target genes (Miska et al, 1999). Therefore any reduction in MEF2 levels should result in the transcriptional repression of their targets in the majority of the contexts, but could also determine the up-regulation of some MEF2 regulated genes if in a particular cell-type/condition the binding to class IIa HDACs is so predominant to transform MEF2 TFs in transcriptional repressors.

In this PhD thesis we aimed to investigate the biological relevance in the mesenchymal lineage of an extreme repression of MEF2 TFs through the over-expression of a super-repressive form of HDAC4. This condition should result in a strong effect that could consist in the acquisition of hallmarks of transformation. In parallel, we describe the cell-cycle dependent regulation of MEF2 TFs and its effect in terms of cellular proliferation.

The results obtained with this PhD thesis should be considered as a starting point to investigate the importance of these two described mechanisms in regulating the malignant conversions of normal cells into tumor ones, allowing a robust and rigorous study of the contribution of the MEF2/class IIa HDACs axis to oncogenesis.

MATERIALS AND METHODS

Cell cultures and reagents.

BJ/Tert cells were cultured in Earl's Salt Minimal Essential Medium (EMEM) (Hyclone) completed with Non-essential aminoacids (NEAA, Hyclone). All other cell lines were grown in Dulbecco modified Eagle medium (DMEM; Lonza). BALB/c 3T3 cells were generated from BALB/c primary MEF using the 3T3 protocol (Todaro et al, 1963). All the mediums were supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100U/ml), and streptomycin (100µg/ml) (Lonza). Cells expressing the inducible form of MEF2 were grown in DMEM (Sigma-Aldrich)/EMEM (Life Technologies) without phenol. For analyses of cell growth, 10^4 cells were seeded, and the medium was changed every 2 days.

The following chemicals were used: 20µM LY (LY294002), 10µM PD9800591, 0.5µM Okadaic acid, Leptomyin B (LC laboratories); 2.5µM MG132, 10 µM BML-210, 1µM 4-hydroxytamoxifen (4-OHT), 10 µM resazurin, 0.5 mg/ml of MTT[3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide],10µg/ml Cicloeximide (CHX), 5µM Roscovitine, 3µM PD0332991, 1µM p38i IV, 1µM Staurosporin, 1mM IPTG, 100 nM Microcystin L1, 50µM ATP, PIC (protease inhibitor cocktail) and dimethyl sulfoxide (DMSO) (all from Sigma-Aldrich); 100nM Torin1 (Cayman); 20µM SKP2in (3-(1,3-benzothiazol-2-yl)-6-ethyl-7-hydroxy-8-(piperidin-1-ylmethyl)-4H-chromen-4-one) (UkrOrgSyntez Ltd.).

Plasmid construction, transfection, retroviral and lentiviral infection, and silencing.

The pEGFPC2, pFLAG CMV5a and pGEX-4T1 constructs expressing MEF2C, MEF2D and SKP2 were generated by PCR and subsequent cloning, using *Eco*RI/*Sal*I restriction sites (NEB). Phospho-defective (Ser-Thr/Ala) and phospho-mimiking (Ser/Asp) MEF2D mutants were generated using Stratagene Quickchange Lightening Kit (Agilent). The deletion mutants of MEF2D and of MEF2D S98A S110A were generated by PCR and cloned into pEGFPC2 and pGEX-4T1 plasmids. pWZL-Hygro-MEF2c-VP16-ER, pWZL-Hygro-MEF2c-ΔDBD-VP16-ER, pWZL-Hygro/pBABE-Puro RAS G12V, pWZL-Neo p53DN (175H), pWZL-GFP, pWZL HDAC4/WT-GFP, pWZL HDAC4/TM-GFP, pWZL-HDAC7-GFP, and pWZL-HDAC7 S/A-GFP were generated by sub-cloning the relative cDNA in pWZL/pBABE back-bones. To generate pWZL-Hygro and pBABE-Puro plasmids expressing SKP2, SKP2DN, SKP2ΔDD (lacking the first 8 aminoacids of the destruction domain), MEF2D-FLAG and MEF2D-S98A/S110A, the relative cDNAs were subcloned into pWZL-Hygro and pBABE-Puro plasmids using a PCR method. The fidelity of all generated plasmids was verified by DNA sequencing. To generate pBABE-Puro-MEF2c-VP16-ER, p-BABE-MEF2c-ΔDBD-VP16-ER, pWZL-Hygro-MEF2c-VP16-ER, and pWZL-Hygro-MEF2c-ΔDBD-VP16-ER, the relative cDNAs were subcloned into pBABE-Puro and pWZL-Hygro plasmids using a PCR method. 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 pcDNA3.1 (EcoRI/ *BamHI* and *BamHI/SalI*). Finally, fragment 1-221 was subcloned into pWZL-HDAC4-TM-GFP restricted by using *Eco-SalI*.

pLKO plasmids (15 and 27) expressing shRNAs directed against hMEF2D were obtained from Sigma-Aldrich. For retroviral infection, HEK293 Ampho Phoenix cells were transfected with 12 µg of plasmidic DNA. After 48h at 32°C, virions were collected and diluted opportunely in order to get the same MOI for all the genes. For lentiviral based knock-down, HEK-293 cells were transfected with 5µg of VSV-G, 15 µg of Δ8.9 and 20µg of

pLKO plasmids. After 36h at 37°C, virions were collected and opportunely diluted in fresh medium. Unless otherwise specified, all transfection experiments in 293 and IMR90-E1A cells were performed with a standard calcium-phosphate method. Silencing of BJ/Tert and BJ/Tert/p53DN were performed with 73 nmoles of SKP2 siRNAs (Invitrogen, sequence: GGUAUCGCCUAGCGUCUGA) and 56 nmoles of p21 siRNAs (Qiagen, sequence: AGACCAGCAUGACAGAUUU). The silencing of human HDAC4 in murine NIH-3T3 was performed with 70 nmoles of HDAC4 siRNAs (Invitrogen).

Immunofluorescence, immunoblotting and immunoprecipitations.

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 antimouse 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.

For immunoprecipitations, cells were collected directly from culture dishes with a rubber scraper into an ipotonic buffer (20mM Tris-HCl, pH 7.5; 2mM EDTA; 10mM MgCl2; 10mM KCl; and 1% Triton-X100) supplemented with protease inhibitors. After lysis, 140mM NaCl was added and lysates were pre-cleared. Next they were incubated with antibody against Ubiquitin, FLAG, GFP, MEF2C or SKP2, followed by protein A beads (GE Healthcare, Little Chalfont, UK).

The following primary antibodies were used: anti-MEF2C C-17 and VP16 (Santa-Cruz Biot.), MEF2C CB (home-made validated antibody), MEF2D (BD Bioscience), ERK, pERK, AKT, pAKT(Ser473), RAN, nucleoporin p62, p120, paxillin, MYC (all from Cell Signaling), SKP2-8D9 (Life Technologies), p21 CP74, FLAG M2 and anti-hemagglutinin (anti-HA) (all from Sigma-Aldrich), RAS (Abcam), GFP (Paroni et al, 2004), HDAC4 (Paroni et al, 2004), CRADD (Henderson et al, 2005), Ubiquitin (Covance).

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 method 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. 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) (Subramanian et al, 2005) 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.

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] (Nurtdinov et al, 2010) 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 (the list of the genes and of the probes are reported below) 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.

203074_at	202455_at	204933_s_at	209147_s_at	213560_at
1552758_at	203003_at	205124_at	209200_at	214684_at
1552760_at	203004_s_at	205659_at	209498_at	216248_s_at
1553272_at	203074_at	206192_at	209926_at	217937_s_at
1554018_at	204225_at	206193_s_at	210226_at	218197_s_at
1555102_at	204621_s_at	206576_s_at	210610_at	218995_s_at
1555629_at	294686_at	207052_at	211143_x_at	219371_s_at
201141_at	204622_x_at	207626_s_at	212099_at	219566_at
202241_at	204885_s_at	207968_s_at	212535_at	219657_s_at
202444_at	204932_at	208328_s_at	213119_at	220266_s_at
222176_at	231242_at	35453_at	38271_at	279_at
222802_at	234393_at	35597_at	38379_at	37711_at
223044_at	236326_at	36082_at	38810_at	35434_at
225140_at	238409_x_at	36214_at	39552_at	32126_at
225641_at	242176_at	37483_at	39821_s_at	988_at
226417_at	1434_at	37611_at	39991_at	39822_s_at
226649_at	1826_at	37623_at	40236_at	34797_at
228813_at	32072_at	37691_at	40922_at	40923_at
229408_at	32123_at	37710_at	41747_s_at	37712_g_at
230658_at	32265_at	37954_at	1380_at	

ANXA8	KLF2	SLC36A1
BHLHE41	KLF3	SLC40A1
CDSN	KLF4	SLC7A2
CEACAM1	MSLN	TNFRSF11B
EDN1	NR4A1	TRIB1
FGF7	NR4A2	
GADD45B	OXR1	
GPNMB	PANK1	
HAVCR2	PPAP2A	
IRS1	RHOB	

Table 2. Upper part: list of the probes used for the bioinformatics analysis. Lower part: List of the MEF2/HDAC4 signature identified through the DNA microarray experiment.

Reporter gene assay.

The promoter of RhoB (300 bp) was cloned from IMR90-E1A 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 12h 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.

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.

Cell-cycle FACS analysis and BrdU assay

For synchronization in G0/G1, NIH-3T3 cells and BJ/Tert cells were serum starved for 48 and 72 hours respectively and then re-activated by addition of FCS. For FACS analysis, cells were fixed with ethanol (O/N), treated with 10µg Rnase A (Applichem Lifescience) and stained with 10mg propidium iodide (Sigma-Aldrich). Data were analyzed with Flowing Software (http://www.flowingsoftware.com/). For S phase analysis, cells were grown for 3h with 100µM bromodeoxyuridine (BrdU). After fixation, coverslips were treated with HCl. Mouse anti-BrdU (Sigma) was used as primary antibody. Nuclei were stained with Hoechst 33258 (Sigma).

Production of recombinant proteins and GST-pull down experiment.

pGEX plasmids expressing the deletion (1-190) of wild-type MEF2D, MEF2D S98A/S110A, MEF2D S98D/S110D, SKP2 and Rb(379-928), (Mendoza-Maldonado et al, 2010), were transformed in BL-21 bacterial cells. Recombinant protein expression was induced with 1mM IPTG at 30°C for 30 minutes and they were purified using Glutathione Sepharose beads (GE Healthcare).

In vitro phosphorylation studies.

Cellular lysates from 2.5 million NIH-3T3 cells for each time-point were obtained. Cells were collected in PBS and lysed for 10minutes in native buffer (HEPES 10mM pH 7,4, Triton 0,1%, $MgCl_2$ 20mM, MnCl 1mM, PMSF 1mM, PIC, NaFl 10mM, $NaVO_4$ 5mM, 0.5 µM Okadaic acid, 100 nM Microcystin L1). 2µg of GST-fusion proteins bound to resin in GST-binding buffer (50mM Tris pH 7.4, 140mM NaCl) were next added. Kinase reaction was conducted by incubating for 1h at 30°C the glutathione bound proteins with cellular lysates supplemented with 50µM ATP and 1µCi γATP (Perkin-Helmer). After several washes, sample buffer was added to the beads. When the recombinant CDKs were used, 250ng of CyclinD1/CDK4 or CyclinE1/CDK2 (Sigma-Aldrich) were incubated with recombinant GST-MEF2D proteins.

Chromatin immunoprecipitation and promoter study

The sequence of p21 CDKN1A proximal promoter (10kb upstream and 10kb downstream from the TSS) was recovered from ENCODE. The presence of putative MEF2 binding site was predicted using CisterZlab (http://zlab.bu.edu/~mfrith/cister.shtml) and JASPAR (http://jaspar.binf.ku.dk/) algoritms.

For each chromatin immunoprecipitation, $2.5x10^6$ cells were used. DNA-protein complexes were crosslinked with 1% formaldehyde (Sigma-Aldrich) in culture medium for 10 min at room temperature. After quenching

with PBS-Glycine 125mM for 5 min, cells were collected and then lysed for 10 min with lysis buffer (5 mM PIPES pH6.8, 85 mM KCl, 0.5% NP40) containing protease inhibitor cocktail (Sigma-Aldrich). Pellets were resuspended in RIPA-100 and sonicated using a Bioruptor UCD-200 (Diagenode), resulting in an average size of 500 bp for genomic DNA fragments. Samples were pre-cleared and immunoprecipitated overnight with 2µg of anti-MEF2C, anti-MEF2C C-17 (Santa Cruz Biotechnology #sc13268), anti-MEF2D (BD Bioscence #610774), anti-GFP (Paroni et al, 2004), anti H3K27ac (Abcam #ab4729) antibodies, or anti-Flag (Sigma-Aldrich #F3165) and pre-immune serum as unrelated control, followed by incubation with protein A blocked with BSA (1g/L) and salmon sperm DNA(1g/l) at 4°C for 2h. Immunoprecipitated samples and inputs were treated with proteinase K solution (1% SDS, 200nM NaCl, 300ug/mL protease 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 90µl of water.

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. *=p<0.05; **=p<0.01; ***p=<0.005.

RESULTS

The forced activation of HDAC4 triggers morphological changes in murine NIH-3T3 fibroblasts and increases their proliferation rate.

Despite the reported pro-oncogenic properties of class I, IIb, III HDACs, a clear demonstration of any involvement of class IIa HDACs in oncogenesis is still missing (Ropero et al, 2007).

In order to investigate any contribution of class IIa HDACs to cellular proliferation and transformation, we chose a common cellular model, the murine NIH-3T3 cells (Todaro and Green, 1963) that, because of mutations in the *INK4* locus, require only one hit to get transformation hallmarks (Aaronson et al, 1970). NIH-3T3 cells were engineered to express the human wild-type form of HDAC4 (NM_006037.3) or a super-repressive mutant, herein referred as TM, in which the three serine required for the binding to 14-3-3 proteins are mutated into alanine (S246A/S467A/S632A) (Grozinger and Schreiber, 2000; Wang et al, 2000). As a matter of fact this mutant is characterized by a prolonged binding to chromatin and by an enhanced repressive capability (Paroni et al, 2007). NIH-3T3 cells expressing oncogenic HRasV12 or GFP were used as positive and negative controls of transformation, respectively. Two independent infections with the TM allele were exploited (TM/i1 and TM/i2). All the over-expressed proteins, with exception for RAS, were GFP-tagged, in order to easily discriminate between the murine and the human ones and for tracking the cells *in vivo* through real time video-microscopy.

The expression of the relative transgenes was checked by means of immunofluorescence (Figure 12A) and immunoblotting (Figure 12B). As demonstrated in Figure 12A, the wild-type form of HDAC4 is mainly cytoplasmic, as subjected to a continuous nucleus-cytoplasm shuttling, while the TM mutant is predominantly nuclear, as expected.

This nuclear retention of the TM mutant enhances its repressive activity as demonstrated with the mRNA levels of *Klf2*, a well-known MEF2/HDAC4 target (Clocchiatti et al, 2013 B) (Figure 12C). Moreover, cells that over-express the super-repressive allele of HDAC4 acquire a spindle-like morphology, they are characterized by a strong reduction in cell size and they lose most of the focal adhesions (characterized by paxillin-positivity) and of the stress-fibers (Figure 12A and 12D). The morphological alterations of the actin cytoskeleton, the reduced cell-size and the reduced number of focal adhesions in HDAC4/TM cells suggest a reduced spreading and adhesion of these cells in respect to the control. This alteration was confirmed by plating the three cell lines on two different substrates: BSA or fibronectin. Figure 12E demonstrates that HDAC4/TM cells are characterized by a decreased spreading under both the conditions.

The described rearrangement of the actin cytoskeleton is characteristic of invasive properties often found in cancer cells. It was previously shown in our lab that the over-expression of HDAC4 in murine fibroblasts increases their random cell motility (Cernotta et al, 2011). The forced nuclear accumulation of HDAC4 (HDAC4/TM) further increases the velocity of the NIH-3T3 cells, confirming the acquiring of invasive properties (Figure 12F). The huge morphological alterations of HDAC4/TM expressing cells are effectively due to the over-expression of this super-repressive allele of HDAC4, as the abrogation of its expression through a siRNA based approach is sufficient to led to a complete reversion of the morphological changes. HDAC4/TM silenced cells are characterized by an increased spreading, rebuilding of stress-fibers and re-organization of the focal adhesions (Figure 12G and 12H); moreover, they lose their invasive properties, scored as random cell motility (data not shown).

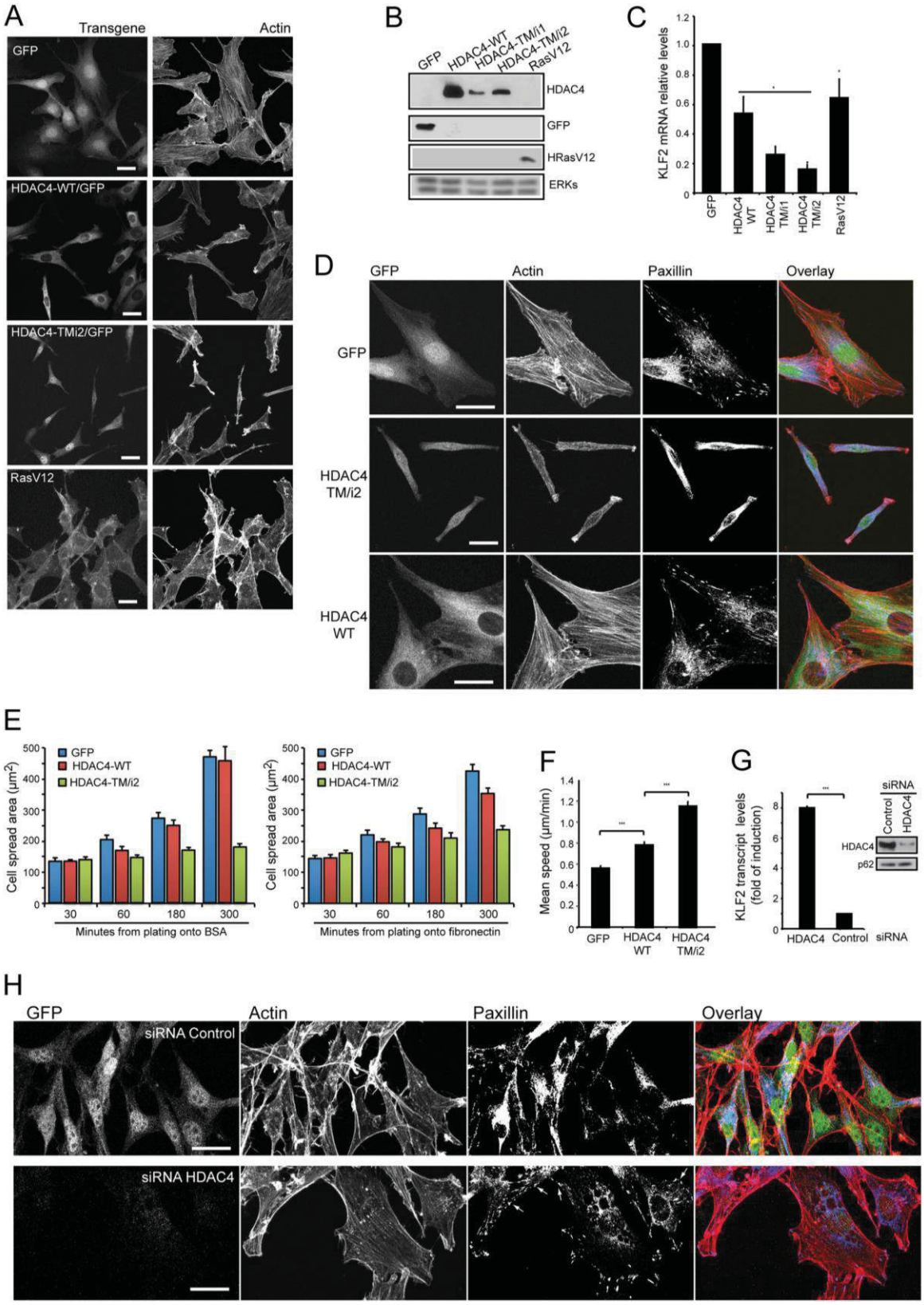


Figure 12 Morphological changes in cells expressing HDAC4/TM. (A) Confocal pictures of NIH-3T3 cells expressing GFP and the different chimeras. Immunofluorescence analysis was performed to visualize HRasV12. AF546-phalloidin was used to decorate F-actin. (B) Immunoblot assays were performed to visualize the different transgenes. The antibodies used were anti-GFP to detect 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. 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. *, $P < 0.05$; ***, $P < 0.001$.

HDAC4/TM over-expressing cells acquire "classical" hallmarks of transformation.

Cells that undergo oncogenic conversion are typically characterized by a loss in cell-adhesion, the acquisition of invasive properties, a reduced population doubling time and the refractoriness to contact-inhibition, an altered cell-cycle profile and metabolism, the acquisition of the capability to grow in absence of anchorage and the capability to form tumors when injected in nude mice (Hanahan and Weinberg, 2000 and 2011). Not all the transformed tumorigenic cells exhibit all these markers, but the latter two are considered to be essential properties of a cancer cell (Hannan and Weiberg, 2011).

In cultivating the HDAC4/TM over-expressing cells we noticed an increase in their proliferation rate, in comparison to HDAC4/WT and GFP expressing cells, and a certain refractoriness to contact inhibition (Figure 13A). In order to assess whether TM-expressing cells acquire also the other classical parameters of transformation, we investigated their ability to form colonies in soft-agar and to form tumors when injected in nude mice. As shown in Figure 13B and C, HDAC4/TM expressing cells, but not GFP ones, developed large colonies in soft agar, similarly to HRasV12-expressing cells, while the over-expression of the wild-type HDAC4 resulted only in few small colonies. These oncogenic properties were confirmed *in vivo* by injecting the engineered NIH-3T3 cells in athimic nude mice. As shown in figure 13D, NIH-3T3 cells over-expressing HDAC4/TM or H-RAS G12V, but not GFP or HDAC4/WT, successfully generated tumors in mice. NIH-3T3 HDAC4/TM overexpressing cells formed nodules becoming palpable 20 days later compared to HRasV12-transformed cells.

The oncogenic properties of the HDAC4/TM allele were confirmed also in another murine mesenchymal cell line derived from BALB/c mice (BALB/c fibroblasts). Also in this case, the nuclear accumulation of HDAC4 is sufficient to acquire the cells the ability to grow in low-anchorage conditions (Figure 13E and F) and to form tumors in nude mice (Figure 13G).

This oncogenic property of HDAC4 in the mesenchymal lineage is shared with HDAC7. In fact, the over-expression in NIH-3T3 cells of the nuclear form of HDAC7 (HDAC7 S/A) in respect to the cytoplasmic wild-type HDAC7 (Figure 13H and I) was characterized by a stronger repression of *Klf2* (Figure 13J), an altered cellular morphology (Figure 13H) and the capability to grow in soft-agar (Figure 13K). These results allow us to suggest that in the mesenchymal lineage class IIa HDACs could have similar and redundant oncogenic properties.

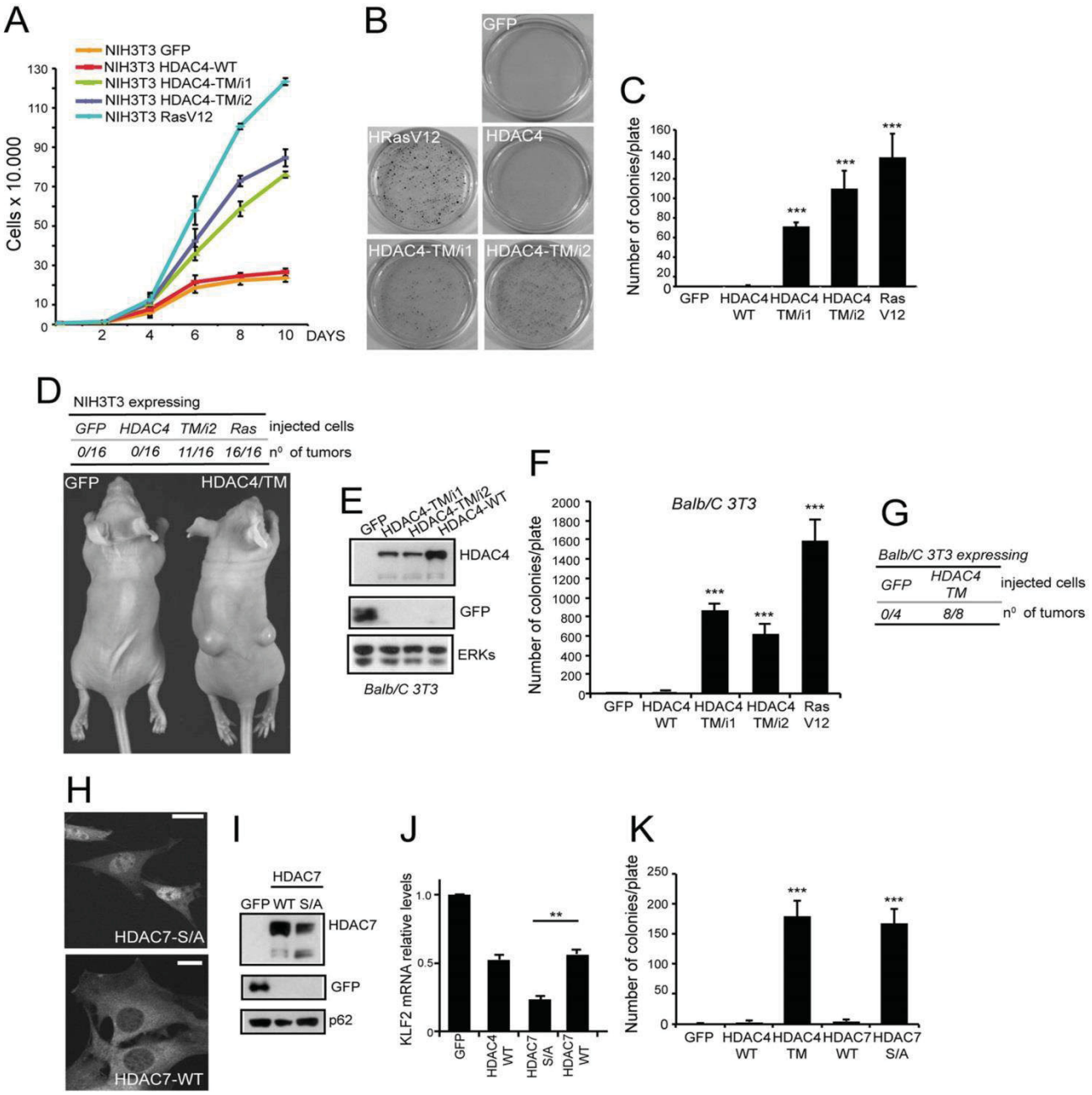


Figure 13 Transforming ability of HDAC4/TM. (A) Cell growth assay in NIH-3T3 cells expressing the indicated transgenes. (B-C) Soft-agar assay pictures (B) and results (C) of NIH-3T3 expressing the indicated transgenes, foci were stained with MTT. (D) Analysis of the tumorigenic properties of NIH-3T3 cells expressing the indicated genes when injected into immunocompromised nude mice. 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 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 µm. (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. **, $P < 0.01$; ***, $P < 0.001$.

Identification of genes under HDAC4 influence.

In order to identify the signature of HDAC4 that determines the transformation of murine fibroblasts, we compared the expressional profiles of cells over-expressing HDAC4/TM or GFP. Furthermore as additional control we also silenced HDAC4/TM expression using a siRNA specific for the human isoform of HDAC4. Through a DNA microarray experiment we identified 47 genes significantly repressed by HDAC4 and 29 significantly up-regulated (Figure 14A and B). For his PhD project we focused the study only on the genes significantly repressed by HDAC4 since, by acting as a repressor, it could be directly involved in modulating their transcription. By means of a Gene Ontology analysis, we figured out that among the genes directly repressed by HDAC4/TM the majority (18%) are involved in the regulation of cell-proliferation and many of them are transcription factors (*Nr4a1*, *Nr4a2*, *Klf2*, *Klf3*, *Klf4*, *Bhlhe41*, *Pbx3*, and *Foxf1a*)(Figure 14C). The microarray data were validated on a sub-set of eleven genes by means of qRT-PCR (data not shown and Figure 16F). As expected, the 80% of the genes repressed by HDAC4 are putative or validated MEF2-targets that have at least a MEF2 binding site in the promoter. Among the panel of the 11 genes selected and validated through qRT-PCR, we included some MEF2 targets (*Klf2, Klf4, Edn1* and *Nr4a1*) and others not previously associated with MEF2 (*RhoB, Nr4a2, Trib1, Anxa8, Irs1, Fgf7* and *Errfi1*), but that have a MEF2 binding site in the promoter. In order to quickly validate the dependence of the transcription of these genes by MEF2, we generated NIH-3T3 that over-express a dominant-negative form of MEF2 (MEF2DN), consisting in the MADS/MEF2 domains of MEF2 without any AD. Except for *Errfi1*, all of the selected HDAC4 target genes were also down-regulated after the expression of MEF2DN (Figure 14D). 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 (*Irs1*, *RhoB*, *Klf4*, *Anxa8*, and *Klf2)* whose expression was influenced by MEF2DN. All of the selected genes were significantly enriched for MEF2 binding in the proximal promoter (Figure 14E). Interestingly, several MEF2 targets identified by us as MEF2-target genes (*Irs1*, *RhoB*, *Klf2*, *Nr4a1*, *Nr4a2*, *Fgf7*, and *Trib1*) have recently been proposed as MEF2 targets in a lymphoblastic cell line by the ENCODE project (Rosenbloom et al, 2010). Among the newly identified MEF2-targets, *RHOB* is particularly interesting for its reported tumor suppressor properties (Jiang et al, 2004). We therefore decided to prove its dependency from MEF2 TFs with a standard luciferase experiment. In particular the proximal promoter (-300 bp to the TSS) of the human *RHOB* containing two putative MEF2 binding sites (Figure 14F) was cloned in the pGL3 backbone and co-transfected together with MEF2C-GFP in IMR90-E1A fibroblasts. As shown in figure 14G, MEF2C was able to activate the transcription of the luciferase gene under the control of *RHOB* promoter at a level similar to the activation induce by the synthetic construct containing a triple repetition of the optimal consensus of MEF2.

The oncogenic activity of HDAC4/TM is largely dependent on MEF2 repression.

MEF2 TFs are the favorite partners of class IIa HDACs (Miska et al, 1999) and the TM mutant of HDAC4, with its prolonged binding to the chromatin, is reported to act as a stronger repressor of MEF2 in respect to the wild-type form (Paroni et al, 2007). However, as explained in the introduction, HDAC4 is able to repress other transcription factors in addition to MEF2 TFs (Clocchiatti et al, 2013 A).

In order to demonstrate the dependency of HDAC4 transforming potential from the conversion of MEF2 TFs into transcriptional repressors, we decided to reactivate MEF2-dependent transcription in HDAC4-transformed 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 (Flavell et al, 2006) (Figure 15A), adopting a strategy already used previously by Olson and Greenberg groups (Arnold et al,

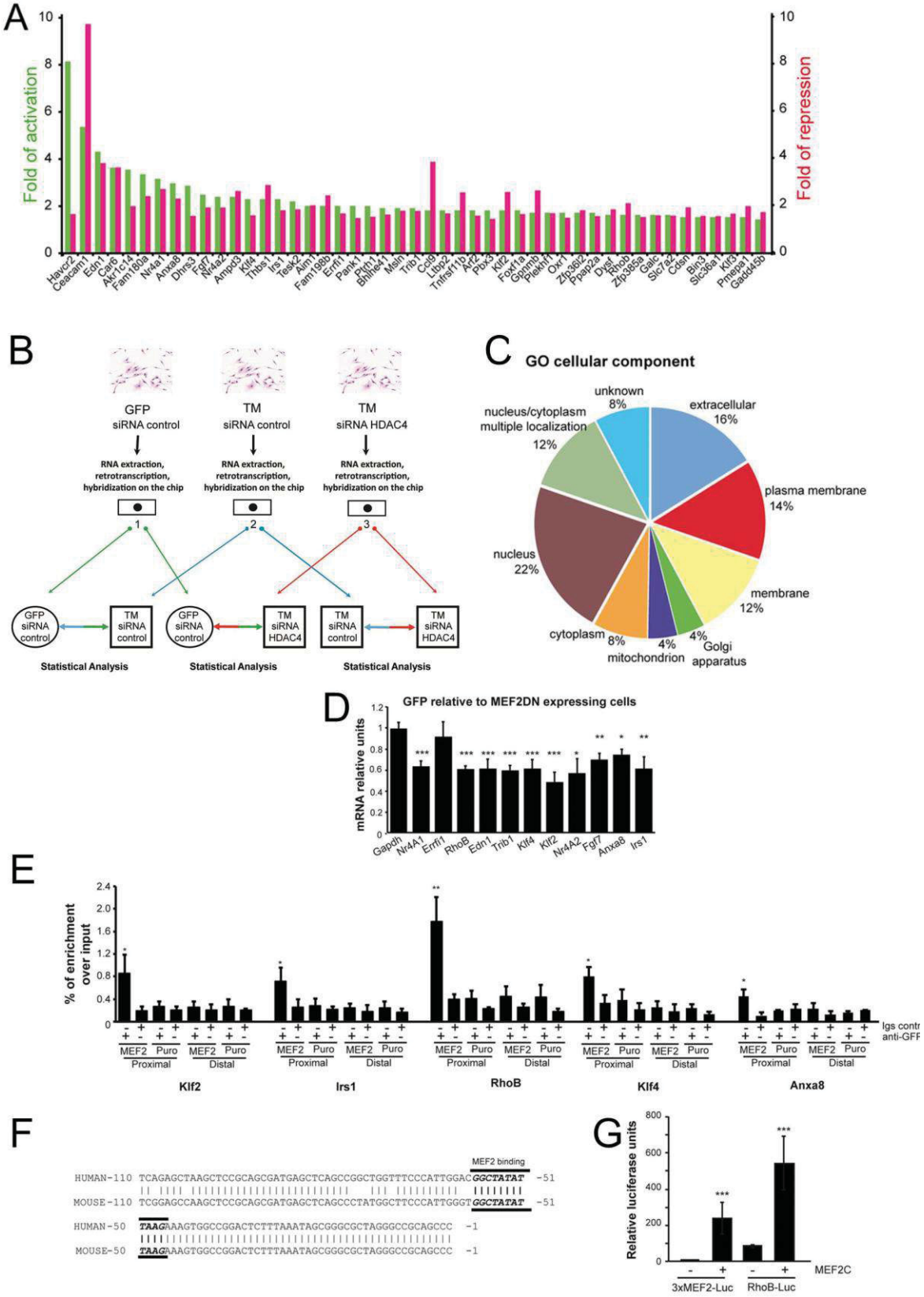


Figure 14 Identification of genes repressed by HDAC4/TM. (A) Diagram representation of the HDAC4/TM target genes. Microarray analyses were performed on GFP- and HDAC4/TM-expressing cells (repressed genes are indicated in red) and in HDAC4/TM cells transfected with control siRNA and the same cells transfected with a siRNA against human HDAC4 (induced genes are indicated in

green). (B) Scheme of the DNA microarray experiment. (C) 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. (D) The mRNA expression levels of 11 HDAC4 target genes were measured using qRT-PCR in GFP- and MEF2DN-expressing cells in order to investigate their dependence from MEF2. (E) Chromatin immunoprecipitation of NIH 3T3 cells overexpressing MEF2-GFP or control Puro. Chromatin was immunoprecipitated with anti-GFP antibody or anti-USP33 (2 μg) 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. (F) Nucleotide sequence analysis of the human and mouse *RhoB* proximal promoters. The MEF2 binding site is underlined. (G) Relative luciferase activity after cotransfection in IMR90-E1A cells of the reporter plasmids pRhoB-Luc (-300/-1 bp) and p3XMEF2Luc, together with MEF2C, as indicated. The Renilla luciferase plasmid was used as an internal control. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

2006; Flavell et al, 2006). We also used as a control the MEF2-VP16-ER lacking the DNA-binding domain (ΔDBD aa 58-86). This construct allows the timely activation of MEF2. In fact, only upon the treatment with tamoxifen the MEF2-VP16 chimera re-localizes to the nucleus (Figure 15B). In NIH-3T3 cells over-expressing HDAC4/TM, the activation of MEF2 following the treatment with tamoxifen is sufficient to reverse the morphological alterations and to promote stress fiber formation and focal adhesion assembling. By contrast, the activation of MEF2-ΔDBD-VP16 was ineffective (Fig. 15C). Similarly, the induction of MEF2 re-activates the transcription of all the tested MEF2-targets *(Klf2, Edn1, Rhob, Irs1)*, but not of *Errfi1* (Figure 15D). As a consequence of abolishing the HDAC4/TM repressive capabilities, these cells lose their proliferative advantage (Figure 15E) and their anchorage-independent growth (Figure 15F).

To further demonstrate 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/TMΔMEF2 (Figure 15G). NIH 3T3 cells were engineered to express GFP, HDAC4/TM-GFP or HDAC4/TMΔMEF2-GFP. HDAC4/TM and HDAC4/TMΔMEF2 mutants have both a sub-cellular localization that is mainly nuclear (Figure 15H) and are expressed in the cells at the same level (Figure 15I). Despite the similar sub-cellular localization and the same expression levels, only HDAC4/TM was able to repress the transcription of *Klf2* (Figure 15J). The repressive incompetence is due to the inability of the TMΔMEF2 mutant to bind MEF2 (Figure 15K) and, as expected, resulted in an almost complete loss of the transforming potential due to the nuclear accumulation of HDAC4 (Figure 15L). Together, these data demonstrate that the strong repression of MEF2 exerted by the TM mutant is a key event for its transforming property.

Several MEF2-target genes repressed by HDAC4 are negatively regulated by the PI3K/Akt pathway

We used our DNA microarray data also to investigate if other common oncogenic pathways were able to converge on MEF2/HDAC4 axis. For doing this, we adopted a GSEA bioinformatics approach (Subramanian et al, 2005). In particular, we compared our DNA microarray data with gene sets from the 3,272 curated MSigDB datasets. From this analysis we found that the phosphatidylinositol3-kinase (PI3K)/Akt/mTOR signature is among the most enriched (PARENT_MTOR_SIGNALING_DN, NES (Normalized Enrichment Score) = -1.47, p=0.039, FDR=0.025). To confirm this result, we used as datasets 91 DNA microarray experiments available on GEO, including different models of transformation in murine fibroblasts and our gene list as a gene set (Figure 16A). The signature of HDAC4 significantly overlaps genes repressed by the PI3K/Akt/mTOR pathway (Figure 16B, C, D, E) through PTEN ablation (Mulholland et al, 2011) (NES=1.46, P<0.005) or TSC2 inhibition (Peña-Llopis et al, 2011) (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 LY294002

(Bromann et al, 2005) (NES=-1.8485, p<0.005), or induced PTEN expression in *Pten* -/- MEFs (Mulholland et al, 2011) (NES=-1.5228, p<0.05).

In order to validate these findings, we treated NIH-3T3 GFP and NIH-3T3 HDAC4/TM-GFP over-expressing cells with the reversible inhibitor of PI3K LY294002 (LY) for 12 and 24 hours and we measured the transcriptional levels of our selected list of 11 genes. Except for *Fgf7*, *Errfi1*, and *Edn1*, the expression of all HDAC4 targets was up-regulated 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 (Figure 16F).

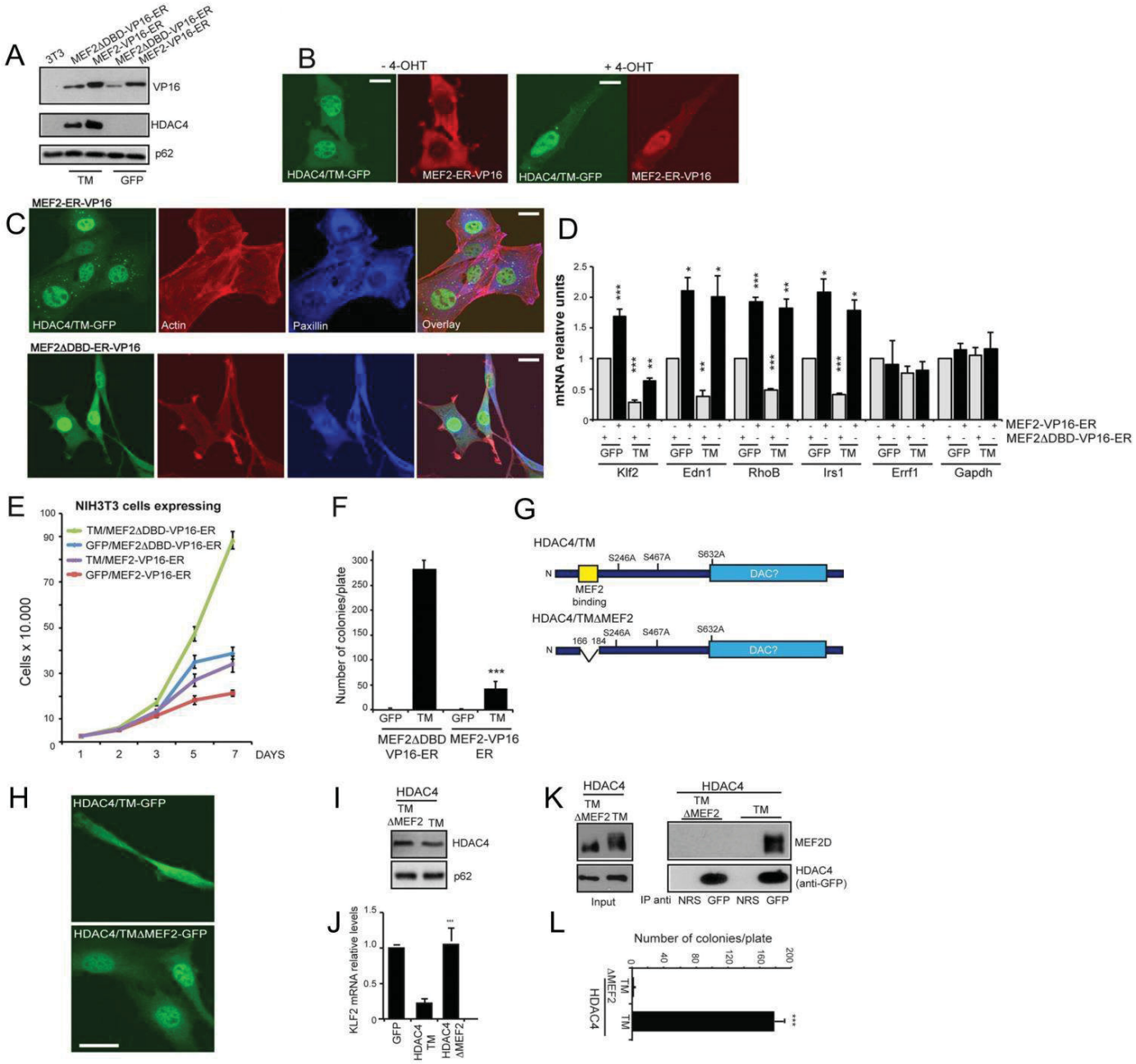


Figure 15 Class IIa HDACs transforming potential is largely dependent on MEF2 repression. (A) Immunoblot analysis of MEF2-VP16-ER levels in NIH-3T3 cells expressing GFP or HDAC4-TM/GFP or control vector (Hygro-Puro). MEF2-VP16-ER-dependent transcription was induced by treating cells with 4-OHT for 24 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 µm. (C) Confocal pictures of HDAC4/TM cells expressing MEF2-ER-VP16 chimera or its mutant defective in DNA binding MEF2ΔDBD-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 µm. (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 MEF2ΔDBD-ER-VP16. (E) Cell growth assay of NIH-3T3 GFP and TM cells expressing the indicated inducible form of MEF2. (F) Quantitative results of colony formation in soft agar of NIH-3T3 cells expressing GFP or HDAC4/TM and the two MEF2 forms. (G) Scheme of HDAC4/TM highlighting the deacetylase domain and the region involved in MEF2 binding. The TMΔMEF2 is deprived of the MEF2 binding domain. (H) Confocal pictures of NIH-3T3 cells expressing HDAC4/TM-GFP or its deleted version for MEF2 binding. Scale bar, 50µm. (I) Immunoblot analysis of HDAC4/TM and HDAC4/TMΔMEF2 levels in NIH-3T3 cells. Immunoblot analysis was performed with anti-GFP antibodies. p62 (nucleoporin) was used as a loading control. (J) 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. (K) Co-immunoprecipitation experiment in NIH-3T3 cells expressing HDAC4/TM or HDAC4/TMΔMEF2. Only the first interacts with MEF2D. NRS, normal rabbit serum. (L) Quantitative results of colony formation in soft agar of NIH-3T3 cells expressing the indicated transgenes. ***, $P < 0.001$.

The PI3K/Akt pathway represses MEF2 transcriptional activity.

The up-regulation of MEF2/HDAC4 target genes upon PI3K/AKT pathway inhibition suggests a putative interference of this pathway or at the level of MEF2 or at the level of class IIa HDACs or could depend on different transcriptional regulators. In order to discriminate between these three hypotheses, first of all we transfected IMR90-E1A fibroblasts (that are easily transfectable) with MEF2C and its synthetic luciferase reporter. As shown in figure 17A, the inhibition of PI3K/AKT pathway is sufficient to significantly up-regulate MEF2 transcriptional activity. Similar results were obtained in NIH-3T3 cells (data not shown). Having demonstrated the not coincidental relationship between PI3K/AKT inhibition and MEF2 activation, we transfected the same cells with a panel of HDAC4 mutants, known in literature for having differential capabilities to repress MEF2, and we scored their susceptibility 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 (Figure 17B). The only exception was the HDAC4 mutant lacking the amino terminus, which is defective for MEF2 binding and thus for repressive activity (Paroni et al, 2007).

The repressive effect of PI3K/Akt signaling pathway on MEF2-dependent transcription was further demonstrated in IMR90-E1A cells expressing a constitutive active (A) version (Myr-Akt) of Akt (Figure 17C). In contrast, a dominant-negative (I) form of Akt (K179M) increased the MEF2C-dependent transcriptional activity (Figure 17C). Similarly to the effect of LY, the repressive influence of HDAC4 was only weakly affected by the coexpression of the Akt variants.

In order to definitively exclude the possibility that the PI3K/AKT pathway could activate MEF2 through the inhibition of class IIa HDACs, we decided to silence simultaneously HDAC4, HDAC5 and HDAC9 in IMR90-E1A cells and measure the effect of PI3K/AKT inhibition on MEF2-dependent luciferase transcription. Transcription from the MEF2 promoter was upregulated 2-fold after PI3K inhibition (Figure 17D). Silencing of class IIa HDAC4/5/9 (Figure 17E) increased the transcription by 4-fold (Figure 17D). Down-regulation 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 17D).

Moreover, the inhibition of PI3K/AKT pathway does not influence the HDAC activity associated to the fraction of class I HDACs in complex with HDAC4 (Figure 17F) and has no effects in terms of HDAC4 or MEF2C sub-cellular localization (Figure 17G and H).

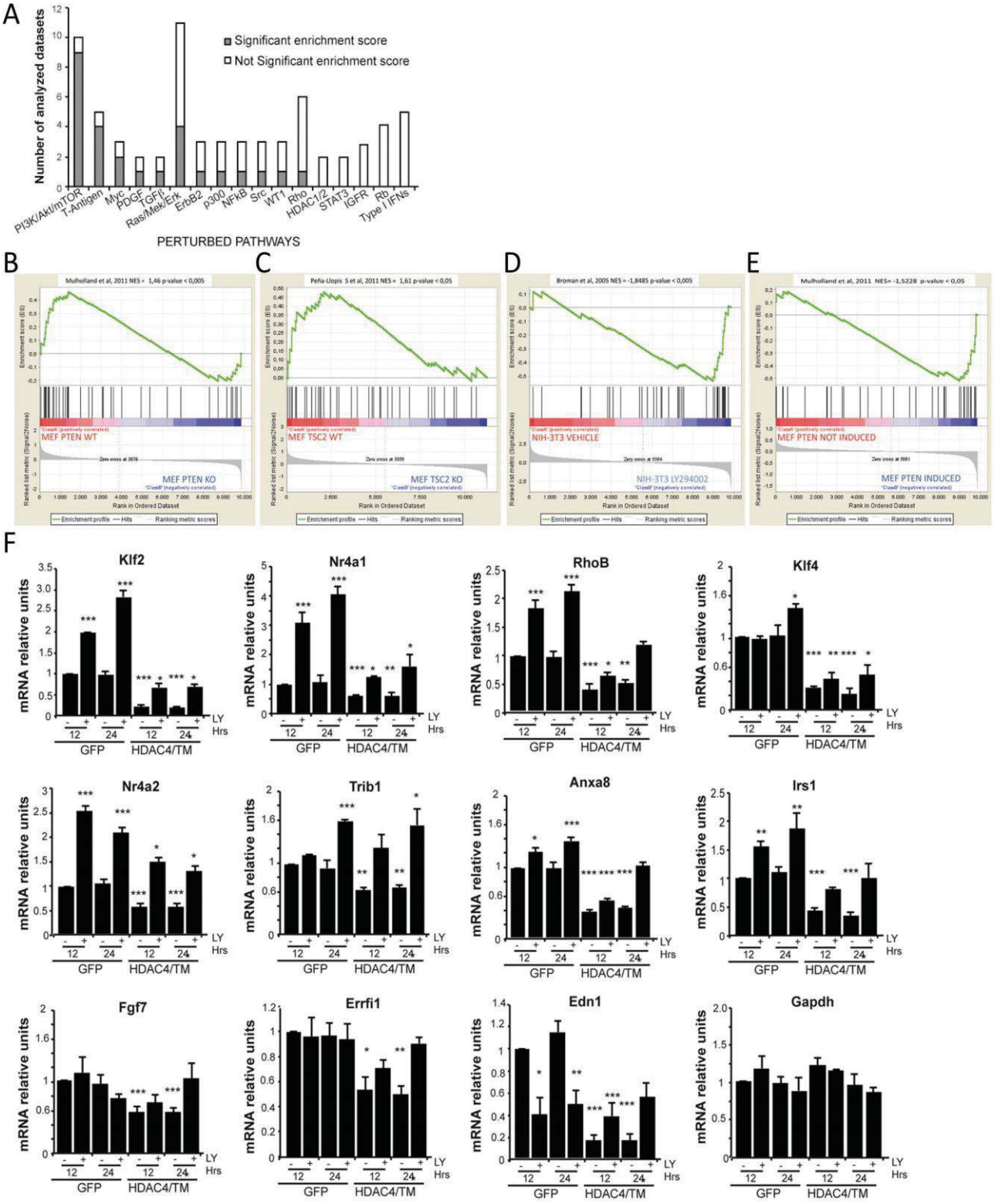


Figure 16 HDAC4/MEF2 signature is also under the influence of PI3K/AKT pathway. (A) Histogram depicting the results of the GSEA analysis conducted on 91 datasets of fibroblasts available on GEO. (B-E) GSEA plots show the enrichment of HDAC4-repressed genes among protein coding genes ranked according to PTEN and TSC2 status and the fold change in LY-treated cells versus control cells. (F) 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.

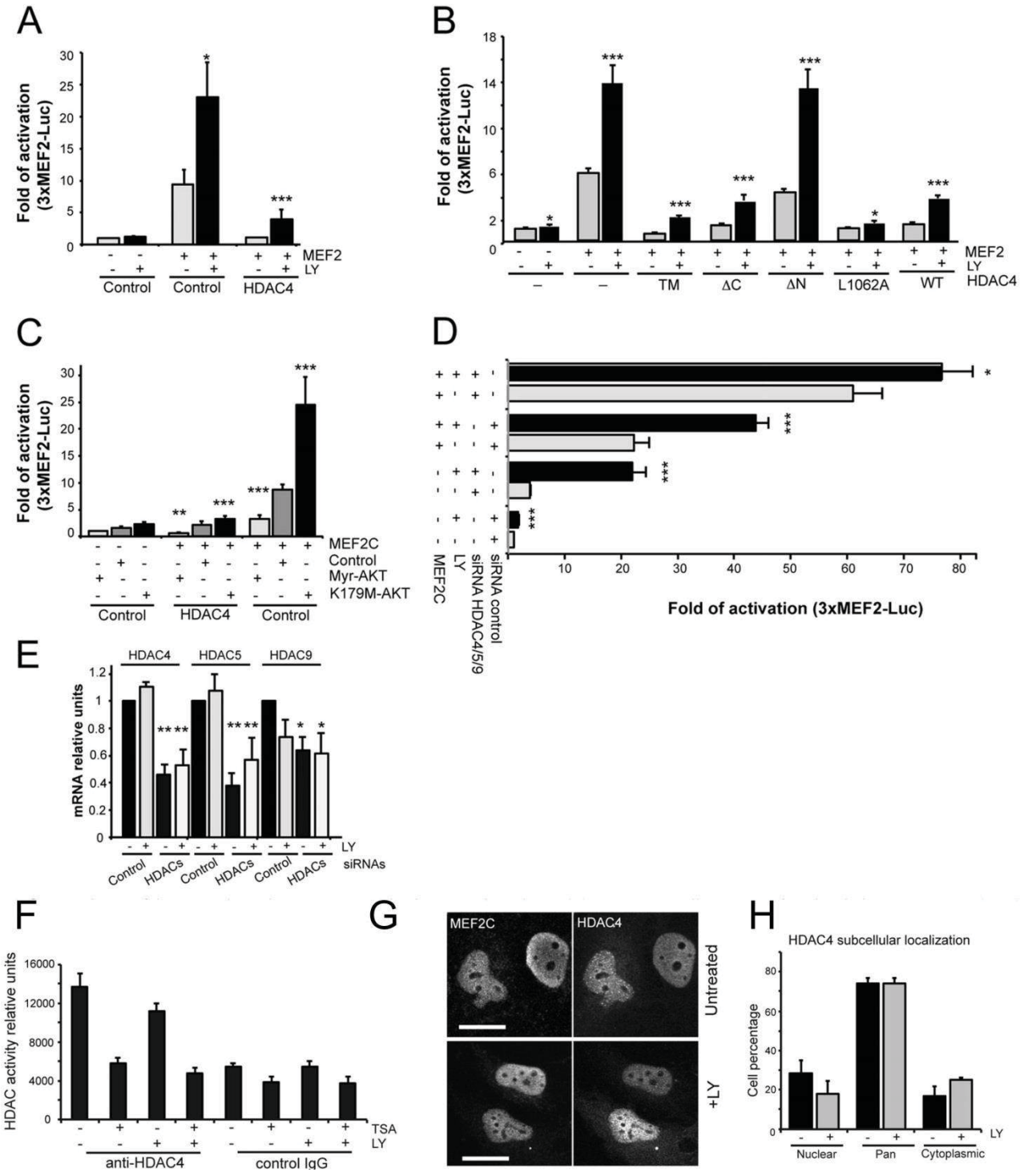


Figure 17 PI3K/AKT pathway directly regulates MEF2 without perturbing class IIa HDACs localization or activity. (A) Luciferase assay on IMR90-E1A cells transfected with the 3xMEF2-Luc (1 μg), the internal control pRL-CMV (20 ng), pcDNA3.1-HA-MEF2C (1 μg), and 300 ng of pEGFP expressing HDAC4. Cells were treated or not for 24 h with LY. (B) Luciferase assay on IMR90-E1A cells transfected and treated as in A, together with the indicated HDAC4 mutants. (C) Luciferase assay on IMR90-E1A cells transfected with the 3xMEF2-Luc (1μg), 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) Luciferase assay on IMR90-E1A cells transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the same amount of a control siRNA and cotransfected after 12 h with 3xMEF2-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. (F) HDAC assay on the fraction of class I HDACs co-immunoprecipitated with HDAC4 in NIH-3T3 cells, treated or not with LY for 18h. TSA (40μM) was used *in vitro* as a negative control of class I HDAC activity. (G) Confocal pictures of IMR90-E1A cells transfected with pcDNA3.1-HA-MEF2C (1μg) 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 μm. (H) 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). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We finally hypothesized that PI3K/AKT pathway could directly impact on MEF2 transcriptional activity or via phosphorylation and inhibition of MEF2 (for example for induced SUMOylation) or by affecting the protein abundance of these transcription factors.

We therefore transfected MEF2C in IMR90-E1A cells and we treated the cells with LY or DMSO. We also co-expressed HDAC4 to evaluate its effect on MEF2C under these conditions. Immunoblot analysis confirmed that HDAC4 influences the electrophoretic mobility of MEF2C (Figure 18A), as already demonstrated by Gregoire and colleagues (Gregoire et al, 2006), probably through its phosphorylation by HIPK2 (de La Vega et al, 2013). Most importantly, we figured out that LY augmented MEF2C protein levels independently from HDAC4 (Figure 18A). These results were confirmed in cells over-expressing the inactive form of Akt, while the hyper-active form of Akt reduced MEF2C levels (Figure 18B).

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 addictive (Figure 18C). These data suggest that the PI3K/Akt pathway impinges on MEF2 by controlling its turnover via the ubiquitin-proteasome system. To sustain this hypothesis, we evaluated MEF2C poly-ubiquitylation in the presence of LY. Co-immunoprecipitation studies in E1A cells, co-expressing MEF2C-GFP and HA-ubiquitin, demonstrated that the PI3K/Akt pathway is required for the poly-ubiquitylation of MEF2C (Figure 18D).

Oncogene dependent regulation of MEF2 activities.

In order to confirm the dysregulation of MEF2 levels due to the activation of PI3K/AKT pathway, we generated NIH-3T3 cells over-expressing a farnesylated form of p110. 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 (Figure 19A). In agreement with this observation, the expression levels of *Klf2, Klf4* and *RhoB* (the strongest MEF2 targets identified in our work) were reduced in cells expressing constitutive active PI3K (Figure 19B). MEF2C and MEF2D were stabilized by the treatment with MG132 also in control cells, demonstrating that MEF2s undergo UPS degradation also in not-transformed cells (Figure 19A).

The transformation of NIH-3T3 cells by RAS destabilizes MEF2C and MEF2D at a level similar to PI3KCA (Figure 19C) and also in this case the consequence of this decreased stability is the repression of *Klf2, Klf4* and *Rhob* (Figure 19D). Finally, introduction of a dominant negative Erk (Erk-CAAX) construct restored MEF2C and MEF2D levels in RAS-transformed NIH-3T3 cells (Figure 19E).

In both the cases, the destabilization and inactivation of MEF2 is coupled to a decrease in MEF2C and MEF2D mRNA levels (Figure 19B and 19D); this could be due to the decreased activity of MEF2 TFs that lose the capability to transcribe themselves (Ramachandran et al, 2008), or could be an independent event that collaborate with the UPS dependent degradation in keeping at low levels MEF2C and MEF2D.

Figure 19A and 19C demonstrate that oncogenes increase the degradation rate of MEF2C and MEF2D. This event seems to be required for reaching a complete transformation as the re-expression of MEF2 in NIH-3T3 p110-CAAX (Figure 19F) and in RAS (Figure 19G) over-expressing cells is sufficient to decrease their transforming potential.

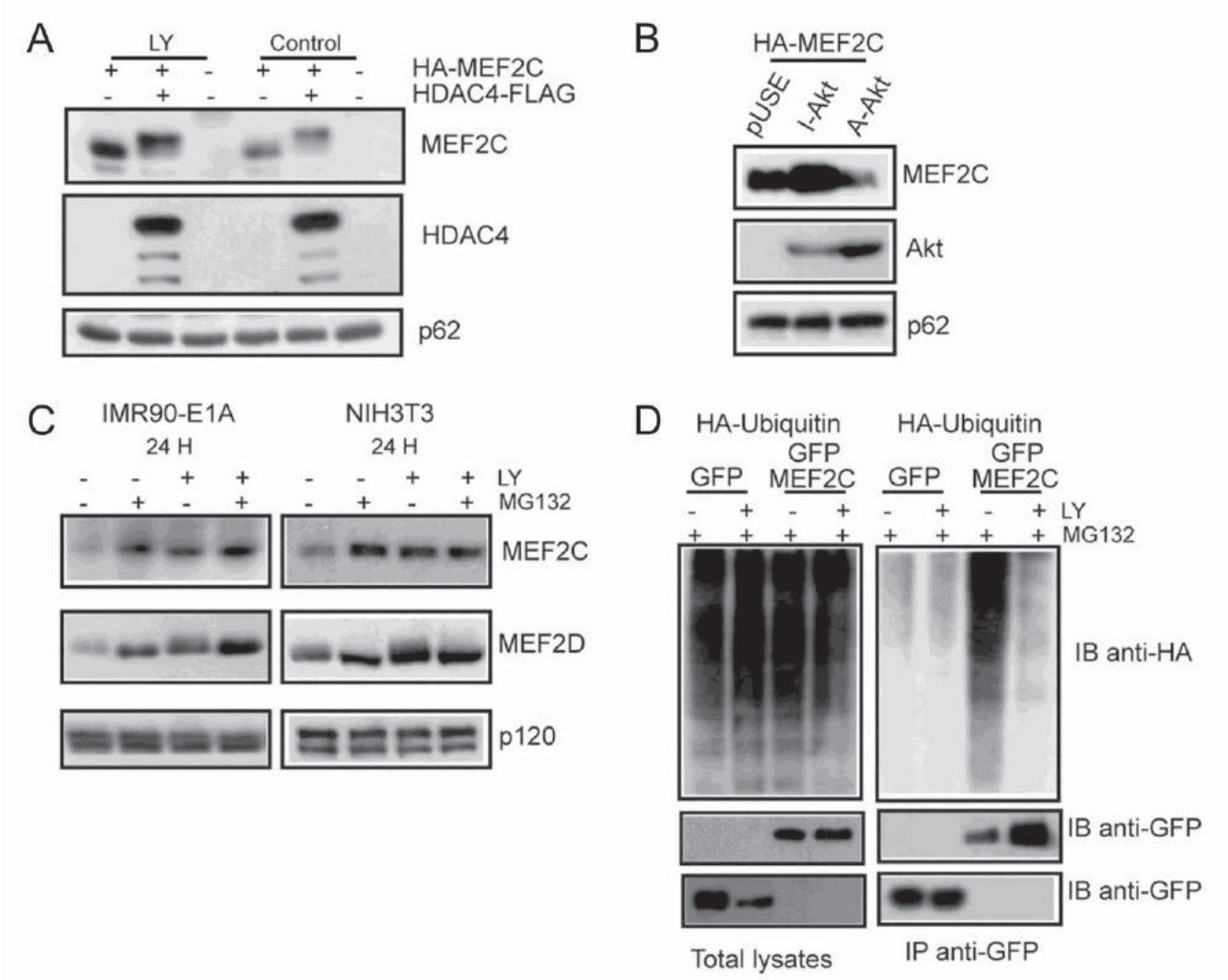


Figure 18 The PI3K/Akt pathway influences MEF2 protein stability. (A) IMR90-E1A cells were transfected with pcDNA3.1-HA-MEF2C(1µg), and 300 ng of pEGFP expressing HDAC4, as indicated. After 12 h, cells were harvested, splitted into two plates and treated with the PI3K inhibitor LY. After 24 h, cellular lysates were generated and subjected to immunoblot analysis using the anti-GFP and the anti-HA antibodies. Nucleoporin (p62) was used as loading control. (B) IMR90-E1A cells were transfected with pcDNA3.1-HA-MEF2C (1µg), and 1µg 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. (C) Immunoblot analysis of MEF2 family members in IMR90-E1A and NIH3T3 cells treated with LY and the proteasome inhibitor MG132 as indicated. p120 was used as loading control. (D) 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 anti-ubiquitin antibody. After being stripped, the filter was probed with an anti-GFP antibody. Inputs have been included.

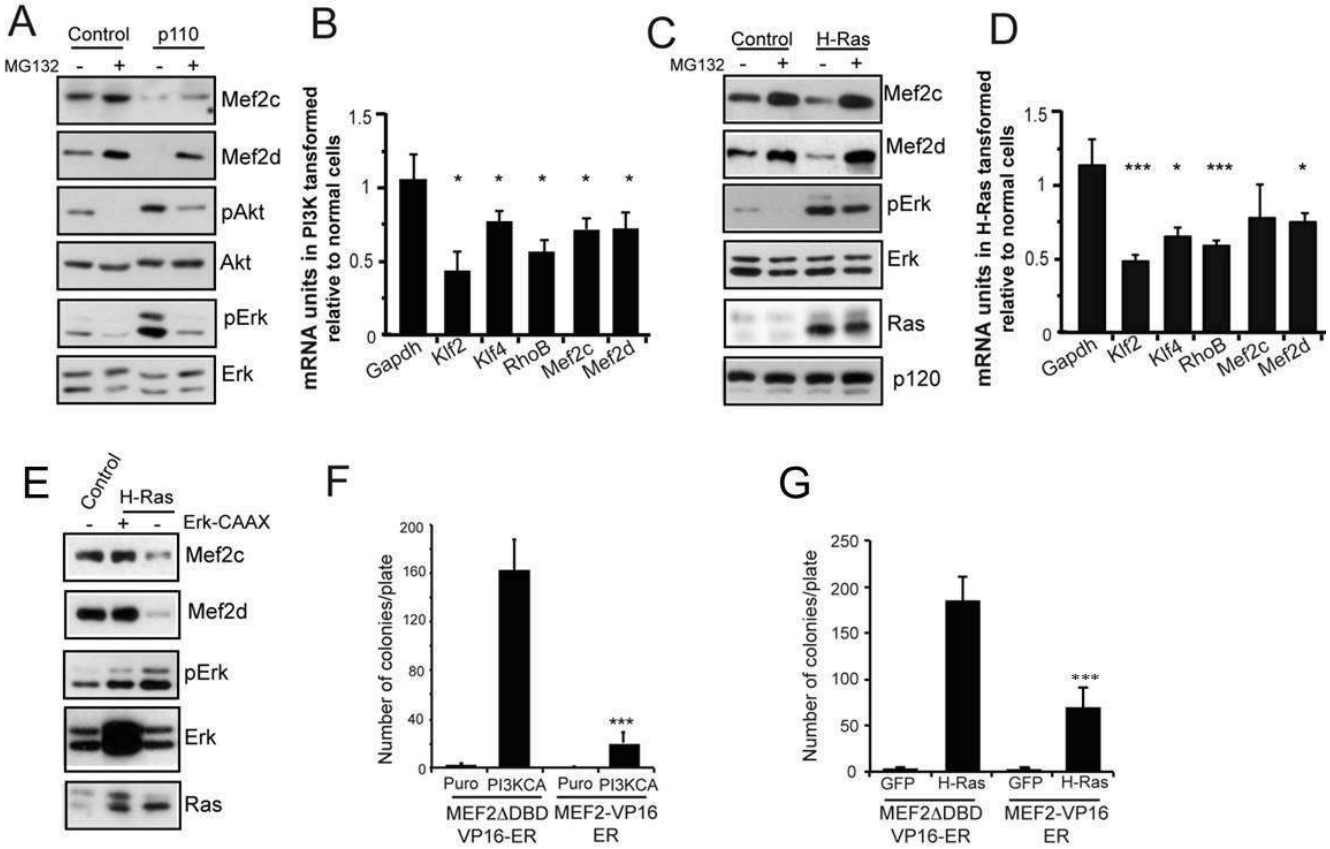


Figure 19 MEF2C/D degradation are required for oncogenic transformation. (A) Immunoblot analysis of MEF2 family members in NIH-3T3 cells stably expressing p110-CAAX or control (PURO) and treated with the proteasome inhibitor MG132, as indicated. The activation of Akt1 and Erk1/2 pathways was assayed with phospho-specific antibodies. Anti Akt1 and Erks antibodies were used as loading control. (B) mRNA expression levels of three MEF2-target genes *(Klf2, Klf4, RhoB), Mef2c* and *Mef2d* in NIH-3T3 p110-CAAX expressing cells were measured using qRT-PCR. mRNA levels were relative to control (PURO) cells. *Gapdh* was used as control gene. Data are presented as mean ± SD; n = 3. (C) Immunoblot analysis of MEF2 family members in NIH-3T3 cells stably expressing H-Ras or control (PURO) and treated for 10h with MG132 as indicated. The activation of Erk1/2 pathway was assayed with the phospho-specific antibody. Anti-Erks antibodies were used as loading control. (D) mRNA expression levels of three MEF2-target genes *(Klf2, Klf4, RhoB), Mef2c* and *Mef2d* in NIH-3T3 H-Ras expressing cells were measured using qRT-PCR. mRNA levels were relative to control (PURO) cells. *Gapdh* was used as control gene. Data are presented as mean ± SD; n = 4. (E) Immunoblot analysis of MEF2 family members in NIH-3T3 cells stably expressing H-Ras or control (PURO) expressing the farnesylated form of Erk1/2 to repress Erk signature, as indicated. The activation of Erk1/2 pathway was assayed with the phospho-specific antibody. Anti-Erks antibodies were used as loading control. (F-G) NIH-3T3 cells expressing the indicated transgenes were seeded in 0.3% top agar and grown for 15 days. Culture medium was changed twice per week. Finally, foci were stained with MTT. Data are presented as mean ± SD; n = 3.

MEF2C and MEF2D protein stability is regulated during the cell-cycle.

Results presented in figure 19 demonstrate that the UPS controls MEF2 protein levels also in non-transformed cells. To explore the regulation of MEF2s during normal cell-cycle, we initially assed MEF2s levels during growth arrest, as induced by density-dependent inhibition. MEF2C and MEF2D levels increase when cells exit the cell-cycle (Figure 20A). In parallel, mRNAs of MEF2-target genes, including MEF2s themselves, rise during density-dependent growth inhibition (Figure 20B). MG132 increases the levels of MEF2C and MEF2D only in growing cells (Figure 20C), demonstrating that the UPS plays a fundamental role in keeping under control the activity of MEF2 TFs in cycling cells.

In order to identify in which phases of the cell-cycle MEF2 TFs are ubiquitylated and degraded, we synchronized NIH-3T3 cells in G0 by serum deprivation; then we re-stimulated the grow of the cells by adding 10% FCS. What comes out is that MEF2C and MEF2D levels decreased as cells entered the S phase (Figure 20D).

Previous studies demonstrated that MEF2s are engaged in the transcription of serum-induced early genes (Suzuki et al, 1995; Han and Prywes, 1995; Kato et al, 1997). Through a real-time PCR experiment on the pool of MEF2 targets identified, we confirmed that at early times from serum addition expression of the MEF2s target genes is augmented (Figure 20E). However, this up-regulation was transient and after 3 hours from stimulation, for *Klf2* and *Klf4*, or 6 hours in the case of *RhoB*, their mRNAs levels were reduced compared to quiescent cells. These results are in agreement with the described down-regulation of MEF2C and MEF2D proteins occurring during late G1/S phase (Figure 20D). As expected, after 12 hours of stimulation, when cells are mainly in S phase, expression of the MEF2s targets was significantly reduced compared to exponentially growing cells (Figure 20E).

Similarly to murine fibroblasts, also in human fibroblasts MEF2 TFs are under a cell-cycle dependent regulation. In particular, in BJ-Tert human fibroblasts bring to contact and to a density dependent arrest, the transcription of the MEF2 targets, *TRIB1* and *RHOB* in particular, is induced in respect to growing cells (Figure 20F). As in murine fibroblasts, this up-regulation is mainly due to the stabilization of MEF2C and MEF2D (Figure 20G), that in quiescent cells are refractory to UPS dependent degradation (Figure 20H). As in murine fibroblasts, also in human cells MEF2C and MEF2D are mainly degraded during G1/S transition (Figure 20I).

SKP2 regulates MEF2C and MEF2D stability.

A key point in the regulation of MEF2C/D during the cell-cycle is their targeting to the UPS. In order to identify the ubiquitin E3-ligase involved in such task, we scrutinized the expression levels of 213 E3-ligases in DNA microarray experiments conducted on fibroblasts quiescent or growing and transformed or not with AKT and RAS. Five E3-ligases come out to be up-regulated both in growing fibroblasts and in RAS/AKT transformed ones: *Skp2, Herc3, Rnf2, Trip12* and *Xiap*. Among them only SKP2 and RNF2 are particularly activated during the S-phase of the cell-cycle (data not shown). We focused our attention on SKP2 (S-phase kinase-associated protein 2). qRT-PCR analysis confirmed the correlation studies of the Bioinformatics analysis; in particular the expression levels of *Skp2* inversely correlate with the MEF2 target genes (*Klf2, Klf4, RhoB*) during density dependent inhibition and in RAS or in PI3K transformed murine fibroblasts (Figure 21A). Furthermore, gene expression analysis in different tumours discovered a significant inverse correlation between the expression of MEF2 target genes and *SKP2* in soft tissue sarcomas, gastric cancer, metastatic skin carcinoma, metastatic melanoma and acute lymphoblastic leukaemia (Figure 21B).

In order to prove the dependency of MEF2 degradation from SKP2, we performed co-expression studies in human fibroblasts expressing the E1A oncogene. The amount of ectopically expressed MEF2C-GFP (Figure 21C) was dramatically reduced in the presence of co-expressed SKP2 and inhibition of the UPS recovered its levels. Also MEF2D-GFP levels were down-regulated by the simultaneous co-expression of SKP2, while a deleted version of the E3 ligase, acting as dominant negative (ΔF-box) (Carrano et al, 1999), efficiently rescued MEF2D-GFP levels (Figure 21D).

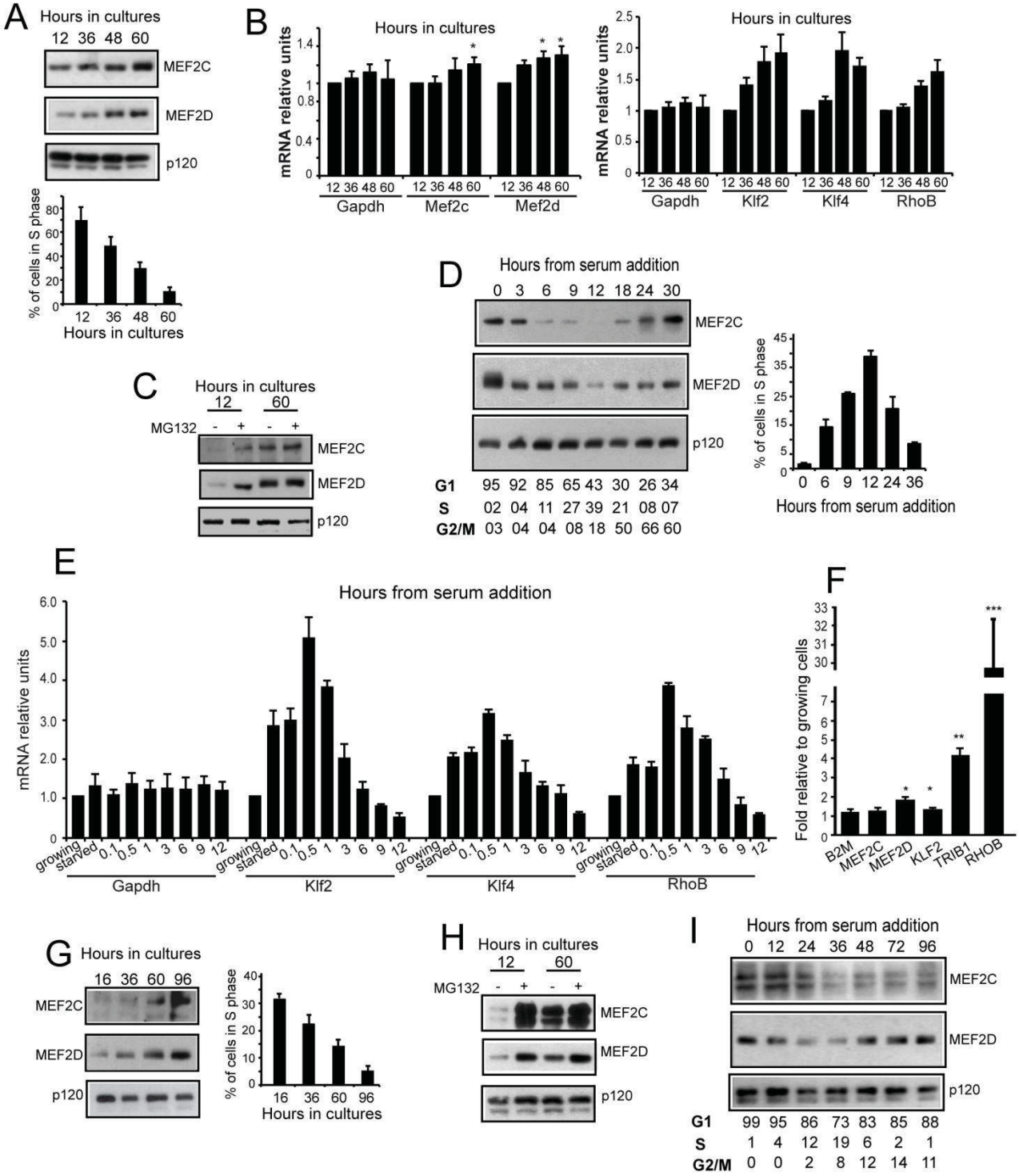


Figure 20 MEF2C and MEF2D levels are regulated during the cell-cycle. (A) Immunoblot analysis of MEF2 family members in NIH-3T3 cells grown for the indicated times in 10% FCS. The fraction of cells synthetizing DNA was scored after BrdU staining. p120 was used as loading control. (B) mRNA expression levels of three MEF2-target genes (*Klf2, Klf4, RhoB*), *Mef2c* and *Mef2d* in NIH-3T3 grown for the indicated times in 10% FCS. mRNA levels are relative to the first time point (12h). *Gapdh* was used as control gene. Data are presented as mean ± SD; n = 3. (C) Immunoblot analysis of MEF2C and MEF2D levels in NIH-3T3 cells grown for the indicated times in 10% FCS and treated or not for 10h with MG132, as indicated. p120 was used as loading control. (D) Immunoblot analysis of MEF2C and MEF2D in NIH-3T3 cells, re-introduced in the growth cycle with 10% FBS after serum starvation for 48 h. Cellular lysates were collected at the indicated time points. Cytofluorimetric analysis of cell-cycle parameters is provided in the lower panel. BrdU positivity is shown in the

histogram. E) mRNA expression levels of three MEF2-target genes (*Klf2, Klf4, RhoB*) in NIH-3T3 collected 12h after the seeding (growing), or grown for additional 48h in 0.5% FBS (starved) and next re-introduced in the growth cycle, with 10% FBS for the indicated times. mRNA levels were relative to growing condition. Gapdh was used as control. Data are presented as mean ± SD; n = 3. (F) mRNA expression levels of three MEF2-target genes *(KLF2, TRIB1, RHOB), MEF2C and MEF2D* in growing BJ/Tert cells (16h) compared to density arrested cells (96h). mRNA levels were relative to the first time point (16h). *β2microglobulin* was used as control gene. Data are presented as mean ± SD; n = 3. (G) Immunoblot analysis of MEF2 family members in human BJ/Tert cells. Cellular lysates were collected at different times after seeding, as indicated. The fraction of cells synthetizing DNA was scored after BrdU staining. p120 was used as loading control. (H) Immunoblot analysis of MEF2 family members in BJ/Tert cells collected at different times after seeding and treated with the proteasome inhibitor MG132, as indicated. P120 was used as loading control. (I) Immunoblot analysis of MEF2 family members in BJ/Tert cells, serum starved for 72h and then reactivated with 10% FBS and collected at different times after the reactivation, as indicated in the figure. Cell-cycle analysis results are provided in the lower panel. Each immunoblot experiment was repeated at least two times with similar results.

In HEK-293 over-expressing SKP2-GFP, the endogenous MEF2D could be immunoprecipitated together with SKP2 and the amount of MEF2D, bound to SKP2, was dramatically increased following MG132 treatment (Figure 21E). Moreover, MEF2D-GFP expressed in 293 cells is poly-ubiquitylated and the co-expression with SKP2 increased its poly-ubiquitylation, whereas the SKP2DN reduces the ubiquitylation (Figure 21F).

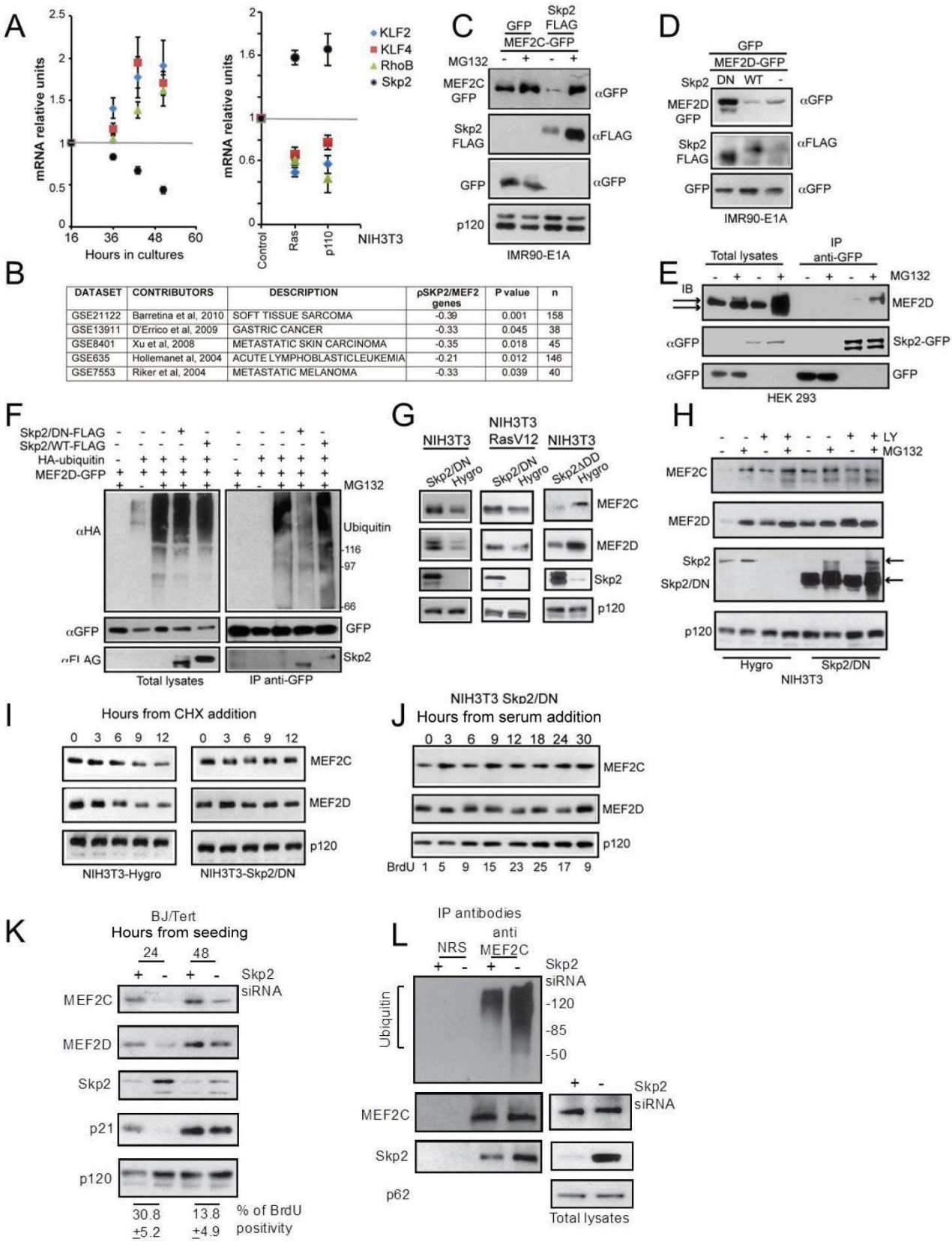
The expression of the dominant negative form of SKP2 is able to rescue the expression of MEF2C and MEF2D both in not-transformed NIH-3T3 and in RAS transformed NIH-3T3 cells (Figure 21G). On the opposite, the expression of the hyperactive form of SKP2 (SKP2ΔDD, Liu et al, 2007 B) causes the destabilization of MEF2C and MEF2D (Figure 21G).

As previously demonstrated, MEF2C and MEF2D levels could be stabilized in NIH-3T3 cells by the treatment with MG132 or the PI3K inhibitor LY294002 (LY). In order to prove that the inhibition of the proteasome or of the PI3K pathway equals to the inhibition of SKP2, we treated NIH-3T3 cells expressing Skp2/DN with LY, MG132 or a combination of both; this treatment failed to further increase the quantities of these TFs, while causes the stabilization of MEF2C and MEF2D in control cells (Figure 21H).

The inhibition of SKP2 by means of SKP2/DN over-expression significantly increases the half-life of MEF2C and MEF2D, assessed by blocking the *de novo* protein synthesis through cycloheximide (CHX). In particular, in proliferating cells a block in protein synthesis elicits a reduction of MEF2C and MEF2D already appreciable at 6 hours from CHX addition. This reduction is abolished in cells expressing SKP2/DN (Figure 21I). Finally, also the down-regulation of MEF2C and MEF2D during G1/S transition was abrogated in SKP2/DN expressing cells (compare figures 21J and 20D). Not surprisingly, these cells exhibited reduced ability of entering S phase after serum stimulation.

To further attest the influence of SKP2 on MEF2 stability, we silenced its expression in human fibroblasts. Down-regulation of SKP2 provoked the up-regulation of both MEF2C and MEF2D proteins (Figure 21K). Furthermore we also proved that poly-ubiquitylation of endogenous MEF2C was reduced in SKP2 silenced cells (Figure 21L). Figure 21L demonstrated also the interaction between endogenous MEF2C and SKP2.

In summary, these results demonstrate that SKP2 could be one of the key E3-ligase involved in the down-regulation of MEF2C and MEF2D during G1/S transition.



DATASET	CONTRIBUTORS	DESCRIPTION	pSKP2/MEF2 genes	P value	n
GSE21122	Barretina et al, 2010	SOFT TISSUE SARCOMA	-0.39	0.001	158
GSE13911	D'Errico et al, 2009	GASTRIC CANCER	-0.33	0.045	38
GSE8401	Xu et al, 2008	METASTATIC SKIN CARCINOMA	-0.35	0.018	45
GSE635	Hollemanet al, 2004	ACUTE LYMPHOBLASTICLEUKEMIA	-0.21	0.012	146
GSE7553	Riker et al, 2004	METASTATIC MELANOMA	-0.33	0.039	40

Figure 21 SKP2 binds and mediates the ubiquitylation of MEF2C/D. (A) mRNA expression levels of MEF2-target genes *(Klf2, Klf4, RhoB)* and of *Skp2* in NIH-3T3 cells grown for the indicated times in 10% FBS or expressing p110-CAAX and H-Ras. The scheme highlights the

inverse correlation between MEF2-targets' expression levels and *Skp2*. (B) Table reporting the inverse correlation between *SKP2* and MEF2 signature in a panel of tumors. (C) IMR90-E1A cells were transfected with pEGFP-N1-MEF2C (1μg), and 2μg of pFLAG-CMV5a SKP2 or pFLAG-CMV5a GFP as control. After 24 hours cells were treated or not with MG132 and after 12h cellular lysates were generated and subjected to immunoblot analysis using the indicated antibodies. p120 was used as loading control. (D) IMR90-E1A cells were transfected with pEGFP-C2-MEF2D (1μg), 2.5μg of pFLAG-CMV5a SKP2, SKP2DN or empty pFLAG-CMV5a, as a control and 200ng of pEGFP-C2. After 36h cellular lysates were generated and subjected to immunoblot analysis using the indicated antibodies. (E) Cellular lysates from HEK-293 cells transfected with 5μg of pEGFP-N1-SKP2 or with empty pEGFP-C2 plasmids and treated or not for 8h with 2.5μM MG132 were immunoprecipitated with an anti-GFP antibody. Immunoblots were performed using the indicated antibodies. (F) HEK-293 cells were co-transfected with HA-ubiquitin (1μg) and MEF2D-GFP (2μg) or GFP and SKP2-FLAG or SKP2DN-FLAG or empty plasmid (4μg). 24h later cells were treated or not for 8h with 2.5μM MG132. GFP fusions were immunoprecipitated using an antibody against GFP and were subjected to immunoblotting using an anti-ubiquitin antibody. After being stripped, the filter was probed with anti-GFP and anti-FLAG antibodies. Inputs have been included. (G) Immunoblot analysis of MEF2 family members and SKP2 in NIH-3T3 cells or NIH-3T3 H-Ras cells stably expressing the dominant negative form (DN), the hyperactive form (ΔDD) of SKP2 or control (Hygro). Immunoblots were performed using the indicated antibodies. p120 was used as loading control. (H) Immunoblot analysis of MEF2 family members and SKP2 (upper arrow) in NIH-3T3 cells stably expressing the dominant negative form (DN, lower arrow) of SKP2 or the control (Hygro) and treated or not for 24h with LY294002 and for 12h with MG132, as indicated. p120 was used as loading control. (I) Immunoblot analysis of MEF2 family members in NIH-3T3 cells stably expressing the dominant negative form of SKP2 (SKP2DN) or the control (HYGRO) and treated for the indicated times with 10μg/ml of CHX. (J) Immunoblot analysis of MEF2 family members in NIH-3T3 cells stably expressing SKP2DN, starved for 48h then reactivated with 10% FBS and collected at different times after stimulation, as indicated. BrdU positivity is shown in the lower part. (K) Immunoblot analysis of MEF2 family members, SKP2 and p21 in BJ/Tert cells transfected for 36h with SKP2 siRNA. Transfection was performed 24h or 48h after the seeding as indicated. P120 was used as loading control. p21 (a well-known SKP2 target) was used as an internal control of SKP2 down-modulation. BrdU positivity is shown in the lower part. (L) BJ/Tert cells were transfected with SKP2 siRNA. After 24h cells were treated or not for 8h with 2.5μM MG132. MEF2C complexes were immunoprecipitated using an antibody against MEF2C and were subjected to immunoblotting using an anti-ubiquitin antibody. After being stripped, the filter was probed with an anti-MEF2C antibody and an anti-SKP2 antibody. Inputs and p62 as loading control have been included.

MEF2 N-terminus domain is involved in the interaction with SKP2.

In order to map the region of MEF2 that is critical for the interaction with SKP2, we initially performed a simple deletion analysis to circumscribe the region entangled. We firstly generated two large truncation mutants, one deprived from the N-terminus and one deprived of the C-terminus. Figure 22A proves that the region 1-117 of MEF2C is sufficient for the interaction with SKP2. Then we moved to MEF2D, that among the MEF2 is the most expressed in fibroblasts (Marinissen et al, 1999). We generated a smaller N-terminus deletion mutant and a deletion fragment of the C-terminus of MEF2D correspondent to the one generated for MEF2C. Putting together the results obtained through this approach, we concluded that the minimal region of MEF2C/D involved in the interaction with SKP2 comprises aminoacids 78-117 (Figure 22B).

In general, many of the proteins regulated through the UPS during the cell-cycle require their phosphorylation as a signal; in particular, SKP2 interacts with its substrates in a phosphorylation-dependent manner (Frescas and Pagano, 2008). In order to identify the residues of MEF2D that must be phosphorylated in order to get its UPS-mediated degradation, we separately mutated into alanine all the serine and the threonine in the first 117 aminoacids of MEF2D that could be good predictive substrates of a kinase: T22, S78, S98, S106 and S110. Neither one of the Ala/Ser or Thr substitutions in MEF2D abrogated its binding to SKP2. However, a slight reduced interaction can be appreciated when serine 98 and 110 were replaced with alanine (Figure 22C). Then, we generated a double phospho-dead mutant of MEF2D in which both serine 98 and serine 110 are mutated into alanine. This mutant is severely impaired in its capability to interact with SKP2 *in vivo* in co-immunoprecipitation experiments and *in vitro* in GST-pull down experiments (Figure 22D and E). As expected, the Ser/Ala mutant is also less ubiquitylated (Figure 22F) and more stable than the wild-type and the phospho-mimetic mutant (Ser/Asp) that is the less stable (Figure 22G). The conformational changes induced by the phosphorylation of these residues are also sufficient for the binding to SKP2 *in vitro* (Figure 22H). Importantly,

the phosphorylation of the same residues, together with others, has been reported to be involved in the destabilization of MEF2C in murine myoblasts (Magli et al, 2010).

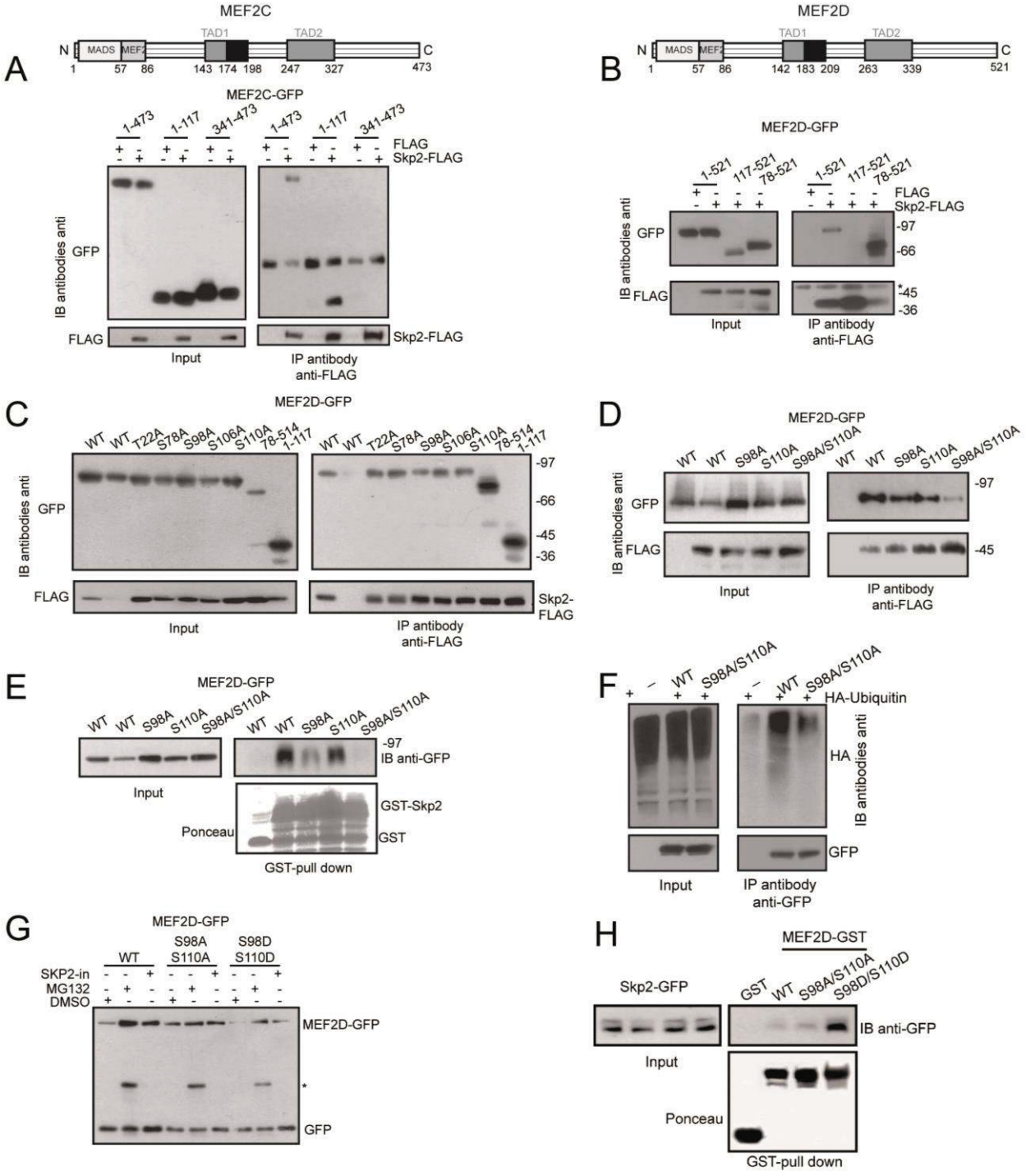


Figure 22 Mapping of SKP2 binding to MEF2C/D. Upper part: Scheme of MEF2C domains. The MADS and the MEF2 domains and the two TADs are indicated. Lower part: Co-immunoprecipitation experiment between MEF2C-GFP deletion and SKP2-FLAG: HEK-293 cells were transfected with pEGFP-N1-MEF2C deletions (1.5µg) and pFLAG-CMV5a-SKP2 or pFLAG-CMV5a (4µg) and treated for 8h with 2.5µM MG132. FLAG fusions were immunoprecipitated using an antibody against FLAG and were subjected to immunoblotting using an anti-GFP antibody. After being stripped, the filter was probed with an anti-FLAG antibody. Inputs have been included. (B) HEK-293 cells were transfected with pEGFP-C2-MEF2D deletions (1.5µg) and pFLAG-CMV5a-SKP2 or pFLAG-CMV5a (4µg). Experimental treatments

and immunopreciptations were as in A. (C) HEK-293 cells were transfected with pEGFP-C2-MEF2D deletions and phospho-dead mutants (1.5µg) and pFLAG-CMV5a-SKP2 or pFLAG-CMV5a (4µg). Experimental treatments and immunoprecipitations were as in A. (D) HEK-293 cells were transfected with pEGFP-C2-MEF2D wild-type and single or double phospho-dead mutants (1.5µg) and pFLAG-CMV5a-SKP2 or pFLAG-CMV5a (4µg). Experimental treatments and immunoprecipitations were as in A. (E) GST-pull down assay using 3µg of recombinant GST-SKP2 or GST alone as a bait and wild-type (wt) or phospho-dead mutants forms of MEF2D-GFP immunoprecipitated from HEK-293 as prey. (F) HEK-293 cells were co-transfected with HA-ubiquitin (2µg) and MEF2D-GFP wild-type or double-phospho-mutant (4µg) or GFP alone and treated or not for 6 h with 2.5µM MG132. GFP fusions were immunoprecipitated using an antibody against GFP and were subjected to immunoblotting using an anti-ubiquitin antibody. After being stripped, the filter was probed with an anti-GFP antibody. Inputs have been included. (G) Immunoblot analysis of MEF2D in IMR90-E1A cells transfected with the wild-type, the phospho-mutant and the phospho-mimiking forms of MEF2D fused to GFP (2µg) and with empty pEGFP-C2 (1µg), used as loading control. After 12h cells were harvested, splitted in three and treated for 12h with DMSO, MG132 and SKP2 inhibitor (SKP2-in), as indicated. (H) GST-pull down assay. Cellular lysates form HEK-293 cells expressing SKP2-GFP were incubated with 2µg of GST, GST-MEF2D, or its phopho-dead and phosphomimetic mutants, as indicated.

MEF2D is a substrate of CDK4.

In order to identify the kinases responsible for the phosphorylation of MEF2D at serine 98 and 110, we adopted an *in vitro* phosphorylation assay using cellular extracts from NIH-3T3 cells. Figure 20A shows that a MEF2D-GST fusion comprising aminoacids 1-190 was phosphorylated *in vitro* and this phosphorylation is augmented when extracts were obtained from cells in G1 phase (cells that re-enter the cell-cycle) compared to quiescent cells (cells serum deprived and blocked in G0). This phosphorylation was reduced when the MEF2D-S98A/S110A double mutant was used (Figure 23A), demonstrating that a kinase, or a group of kinases, active in G1/S are involved in the specific phosphorylation of serines 98 and 110. Since these serine residues share consensus phosphorylation sequences with several S/T-P kinases (Erks, mTOR, GSK3β, p38, CDKs), we tested whether the relative specific inhibitors could influence MEF2D phosphorylation *in vitro*. We left out GSK3β, because it is less active in G1/S and for the same reason CDK1 that is more active in mitosis. Staurosporine was used as positive control. Only mTOR, p38 and CDK4 specific inhibitors reduced MEF2D (1-190) phosphorylation in our *in vitro* assay (Figure 23B). By comparing the results obtained with the wild-type form with the ones obtained with the phospho-dead mutant of MEF2D as substrates it could be possible to identify the kinase involved in the phosphorylation of these serines. The experiments conducted with the MEF2D-S98A/S110A mutant demonstrated that p38 and mTOR are not involved in the phosphorylation of these residues, as both the p38 and the mTOR inhibitors efficiently repressed also the phosphorylation of the double mutant; on the contrary the CDK4 inhibitor was less efficient on the phospho-dead mutant in respect to the wild-type, suggesting the involvement of CDK4 in the phosphorylation of MEF2D at those sites (Figure 23C). In order to demonstrate this finding, we performed an *in vitro* phosphorylation experiment using the complex CDK4/CyclinD1 as a kinase source and as substrates the wild-type and the phospho-dead forms of MEF2D. As a negative control we used the CDK2/CyclinE complex that should not be able to phosphorylate MEF2D as reported in figure 23B, where we used Roscovitine to inhibit CDK2. As shown in figure 20D, CDK4/CyclinD1 was able to phosphorylate MEF2D *in vitro* and this phosphorylation was reduced when the S98A/S110A mutant was used, while CDK2/CyclinE was not effective both on MEF2D and MEF2D S98A/S110A. The efficacy of both the kinases was tested on GST-Rb (data not shown). The dependence of MEF2 activity from CDK4/CyclinD1 was previously identified independently from two other labs, but the phosphorylation event beyond the regulation of MEF2 activity was never investigated in details (Lazaro et al, 2002; Anders et al, 2011).

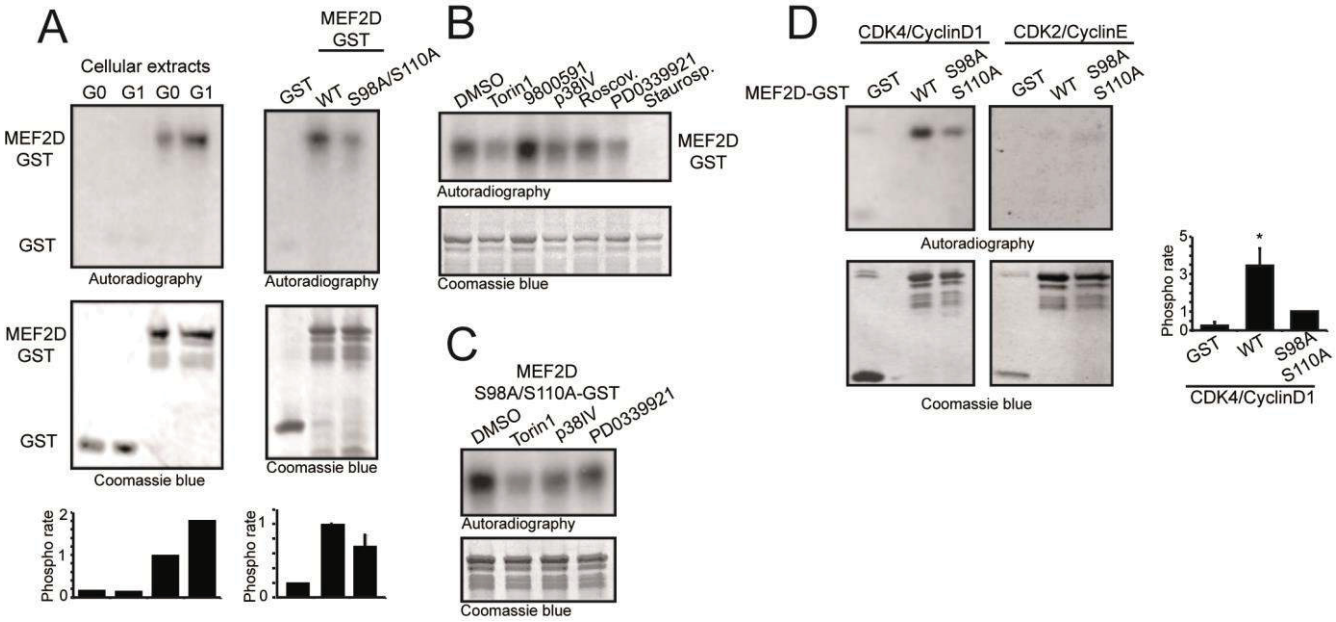


Figure 23 MEF2D is phosphorylated by CDK4/CyclinD1. (A) Left panel: Autoradiography after *in vitro* phosphorylation of MEF2D-GST and GST as control, using cellular extracts from NIH-3T3 cells serum starved and incubated or not for 4h with 10%FCS. Right panel: Autoradiography after *in vitro* phosphorylation of wt and phospho-dead MEF2D-GST and GST as control, using cellular extracts from NIH-3T3 cells serum starved and next treated for 4h with 10%FCS. Coomassie staining was used as loading controls.
(B-C) Autoradiography after *in vitro* phosphorylation performed on GST-MEF2D (B) and the phosphodead form (C) in the presence of the indicated kinase inhibitors or DMSO, using cellular extracts from NIH-3T3 cells serum starved and next treated for 4h with 10%FCS (These cells are enriched in late G1-early G1/S fractions). Coomassie staining was used as loading controls. (D) Autoradiography after *in vitro* phosphorylation performed on GST-MEF2D and GST-MEF2D S98A/S110A, using recombinant complexes of CyclinD1/CDK4 or CyclinE1/CDK2 in their physiological ratio.
Coomassie staining was used as loading controls. Densitometric analysis is also provided.

Roles of MEF2s in the regulation of cell-cycle progression.

Considered the cell-cycle dependent degradation of MEF2, we hypothesized that it should be necessary for the progression through the cell-cycle. Firstly, we investigated the effects of MEF2 hyper-activation and MEF2 repression on NIH-3T3 cellular proliferation. In order to activate MEF2 we used the MEF2-VP16-ER chimera and, as a control, the MEF2-VP16-ER lacking the DNA-binding domain (ΔDBD aa58-86). To get MEF2 repression, we used a strong dominant-negative form of MEF2 in which the DNA binding domain of MEF2C is fused to the Engrailed repressor (MEF2-ENG) (Arnold et al, 2007). NIH-3T3 cells expressing the MEF2-VP16 showed reduced proliferation, reduced incorporating of BrdU and increased number of cells in the G1 phase of the cell-cycle (Figure 24A/B/C). On the contrary, cells expressing the repressive version of MEF2 exhibit increased proliferation, highest percentage of BrdU incorporation and reduced number of cells in G1 (Figure 24D/E/F). We also investigated the capability of NIH-3T3 cells expressing the different MEF2 versions to re-enter the cell-cycle after serum starvation. As a positive control we used cells expressing SKP2ΔDD, the hyperactive version of the E3 ligase. As expected, the cells expressing the hyperactive SKP2 did not enter G0 in response to serum starvation (Figure 24G). In the presence of the repressive form of MEF2, a discrete amount of cycling cells can still be detected also after serum starvation (approximately 30% of the cells in S and G2 phases) (Figure 24G). In respect to the cells expressing the GFP and ΔDBD form of MEF2, cells expressing the hyper-active form of MEF2 enter with a significant delay the S phase (Figure 24G). Not surprisingly, NIH-3T3 cells overexpressing the wild-

type degradable form of MEF2 proliferate more in respect to the NIH-3T3 overexpressing the not phosphorylable form of MEF2D and the latter population is quickly lost in a competition assay (Figure 24H).

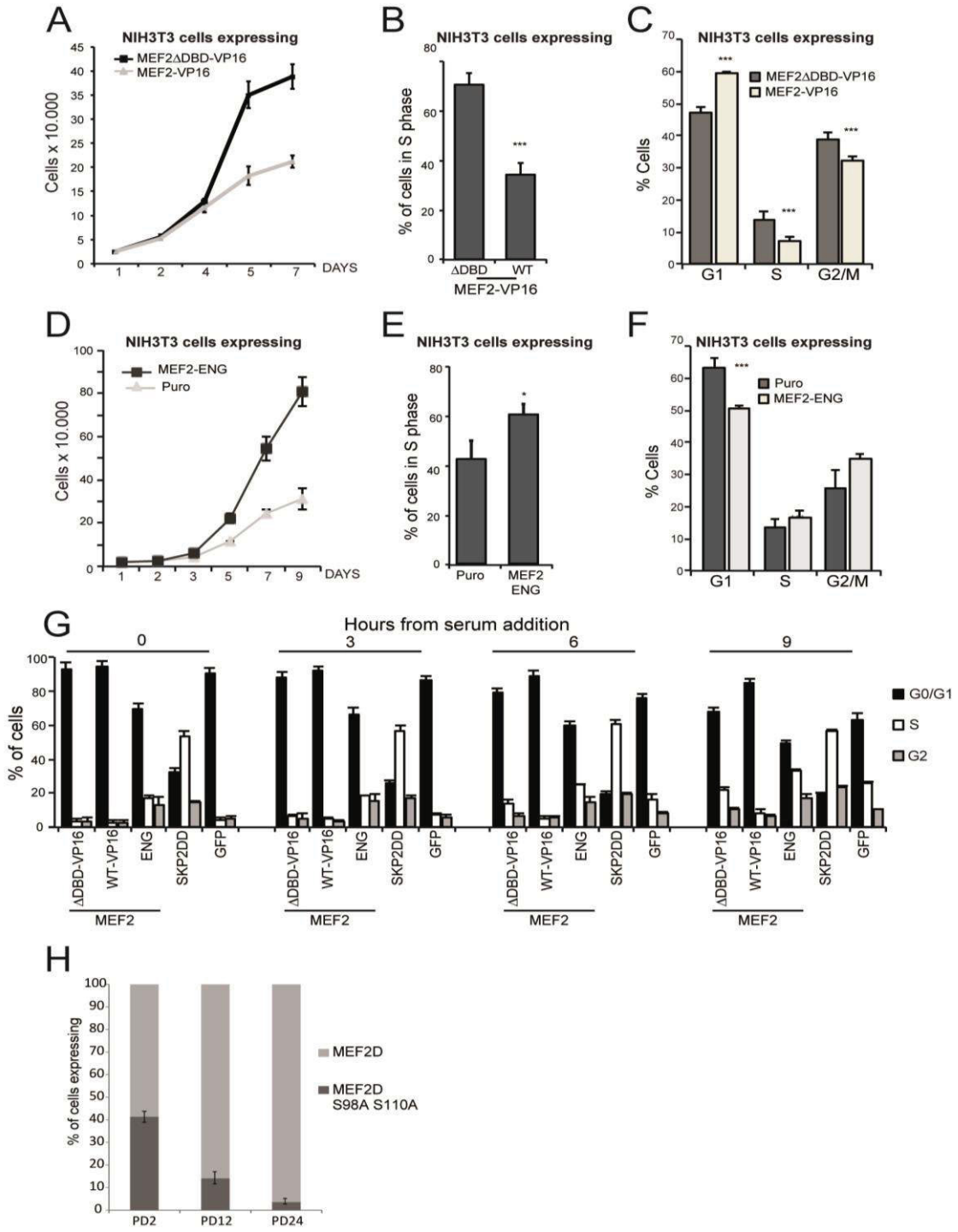


Figure 24 MEF2 affects the proliferation of NIH-3T3 fibroblasts. (A) Cell-growth assay on NIH-3T3 cells expressing the two transgenes and treated with 4-OHT for the indicated times. Data are presented as mean ± SD; n = 3. (B) Quantification of BrdU positivity of NIH-3T3 cells expressing the indicated transgenes and treated with 4-OHT for 48h. Data are presented as mean ± SD; n = 5. (C) Cell-cycle profile of NIH-3T3 cells expressing the indicated transgenes and treated with 4-OHT for 48h. Data are presented as mean ± SD; n = 4. (D) Cell growth assay in NIH-3T3 cells expressing the two transgenes and grown for the indicated times. Data are presented as mean ± SD; n = 3. (E) Quantification of BrdU positivity in NIH-3T3 cells expressing the indicated transgenes 48h after the seeding. Data are presented as

mean ± SD; n = 5. (F) Cell-cycle profile of NIH-3T3 cells expressing the indicated transgenes. Analysis was performed 48h after seeding. Data are presented as mean ± SD; n = 4. (G) Cell-cycle profile of NIH-3T3 cells expressing the indicated transgenes, serum starved (time 0) or after different hours from serum addition. SKP2DD was used as positive control of unrestricted proliferation. Data are presented as mean ± SD; n = 3. (H) Competition assay: NIH-3T3 cells expressing the relative MEF2D forms were co-cultivated for 24 population doublings (PD) and the relative abundance of the two species was scored by looking at the cDNA levels of the two isoforms with specific primers. Data are presented as mean ± SD; n = 3.

The negative impact of MEF2 on cellular proliferation and on the progression through G1/S checkpoint could be easily explained by the direct regulation of the transcription of a CDK inhibitor (CDKi). We looked at our DNA microarray experiment and we checked if any CDKi of INK4 or Cip/Kip families were significantly repressed by HDAC4. Among the main CDKi, only *p21 Cdkn1a* was significantly repressed by HDAC4 (Figure 25A). HDAC4 is reported to negatively affect *p21* transcription through the engagement of multiple transcription factors, among the others Sp1 and Foxp3 (Wilson et al, 2008; Mottet et al, 2009; Liu et al, 2009). We hypothesized that in addition to these TFs, class IIa HDACs could regulate *p21* transcription also through the repression of MEF2 TFs. In order to demonstrate a direct involvement of MEF2 TFs in *p21* transcription, we measured the mRNA levels of *p21 Cdkn1a* in NIH-3T3 cells expressing the hyper-active or the super-repressive forms of MEF2. As shown in Figure 25B the activation of MEF2 induces the up-regulation of *p21* levels, while its repression causes the down-regulation of *p21* at a level similar to the levels reached by the other MEF2 target genes. This transcriptional up-regulation of *p21* was confirmed at the protein levels, as shown in Figure 25C. *p21* has a similar transcriptional behaviour to the other MEF2 targets in response to serum starvation and serum re-addiction (compare Figure 25D and Figure 20E).

A

Cdks inhibitors	Ratio in cells expressing HDAC4-GFP versus GFP	p-value
Cdkn1a (p21)	0,6141	2,7677E-02
Cdkn1b	1,1379	3,2716E-01
Cdkn1c (p57)	0,8345	8,8199E-01
Cdkn2a	1,0349	6,7010E-01
Cdkn2b (p15)	1,1075	4,8153E-01
Cdkn2c (p18)	0,9935	9,6692E-01
Cdkn2d (p19)	1,2106	1,9732E-01
Cdkn3	0,9801	9,1779E-01

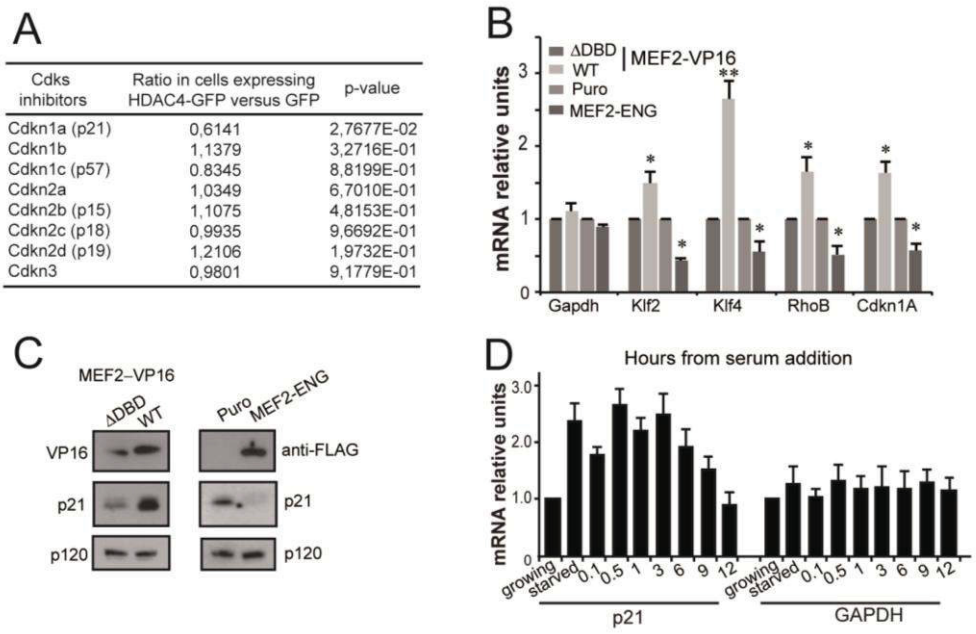


Figure 25 MEF2 regulates *p21 CDKN1A* transcription in murine fibroblasts. (A) Table showing the mRNA fold inductions of the indicated CDK-inhibitors, by comparing their levels of expression in NIH-3T3 expressing HDAC4-GFP or GFP as control. (B) mRNA expression levels of MEF2-target genes *(Klf2, Klf4, RhoB)* and *Cdkn1a* in NIH-3T3 cells expressing the indicated transgenes and collected 36h after the seeding. *Gapdh* was used as control. Data are presented as mean ± SD; n = 3. (C) Immunoblot analysis of p21/CDKN1A levels in NIH-3T3 cells expressing the indicated transgenes. Anti-VP16 and anti-FLAG antibodies were used to reveal the expression of the transgenes. p120 was used as loading control. (D) mRNA expression levels of *Cdkn1a* in NIH-3T3 cells treated as in 20E. *Gapdh* was used as control.

Similarly to the results obtained in murine fibroblasts, also in human fibroblasts the activation of MEF2-dependent transcription using the MEF2-VP16-ER chimera dramatically suppressed cell proliferation and DNA

synthesis (Figure 26A/B) and, of course, up-regulates the transcription of well-known MEF2-targets, including *p21* (Figure 26C/D), in spite of the p53 back-ground of the cells. The up-regulation of *p21* was confirmed also by immunoblot (Figure 26E) and was independent from p53 (Figure 26C/D/E).

To further demonstrate the negative impact of MEF2 on cell-cycle progression and its role in the transcription of *p21*, we knocked-down MEF2D expression through a lentiviral infection. In BJ/Tert/p53DN cells, MEF2D down-regulation was coupled with the reduction of CDKN1A levels, an increase in DNA synthesis, an augmented cell proliferation and a reduction of the mRNA levels of the MEF2-target genes, with similar results by using two different shRNAs (Figure 26F/G/H/I).

Similar results were obtained in BJ/Tert/E1A/RAS; also in this case the down-regulation of MEF2D causes a decrease in the levels of CDKN1A/p21 (Figure 26J), an increase in cell proliferation (Figure 26K/L) and the down-regulation of the MEF2 targets (Figure 26M).

CDKN1A is a key element in the anti-proliferative activity of MEF2.

To quantify the effective contribution of CDKN1A to the anti-proliferative signalling of MEF2s, we silenced its expression in BJ/Tert and BJ/Tert/p53DN cells expressing the inducible MEF2 or its mutant deleted in the DNA binding domain (Figure 27A). The anti-proliferative effects of MEF2, as well as the inhibition of DNA synthesis elicited by MEF2 up-regulation were almost entirely abrogated when CDKN1A was down-regulated (Figure 27B and 27C).

In order to find a molecular link between SKP2, MEF2 and p21, we took advantage from a recently characterized inhibitor of SKP2 (SKP2i), which displays anti-proliferative effects especially due to the stabilization of p21 and p27 (Chan et al, 2013). At least part of the cytostatic effect of SKP2i is due to MEF2 stabilization, as the contemporary inhibition of SKP2 and knock-down of MEF2D limits the effectiveness of the inhibitor (Figure 27D and E), probably because of the decreased transcription/stabilization of p21 (Figure 27F).

MEF2 TFs bind the first intron of p21 CDKN1A and promote the acetylation of H3K27, thus enhancing p21 transcription

The dependence of *p21* transcription from MEF2 could also be indirect and rely on the induction of some MEF2 targets, such as *KLF4* and *KLF2*, that are reported inducers of p21 (Chen et al, 2001; Wu et al, 2004). To clarify this point, we inspected the genomic region around the *CDKN1A* transcription start site for the presence of MEF2-binding consensus sequences. Figure 28A schematizes the organization of the *CDKN1A* genomic region and highlights the presence of 6 putative MEF2-binding sequences in the promoter and in the first intron of *CDKN1A* gene. In order to map the binding of MEF2 TFs to *p21* genomic regulative regions, we performed ChIP experiments on BJ/Tert and BJ/Tert-P53DN cells using two different anti-MEF2C antibodies and an antibody against MEF2D (Figure 28B). Both MEF2C and MEF2D were found out to bind the CDKN1A genomic region with the highest enrichment for the MEF2-consensus sequence at position +2.1kb (Figure 28B). This enrichment was comparable to the positive control, the region containing the MEF2-binding sequence of the *RHOB* promoter.

The genomic region of *CDKN1A* bound by MEF2 was previously characterized by the ENCODE project (Dunham et al, 2012) as enriched in histone H3 lysine 27 acetylation (H3K27ac), a marker of active enhancers (Zentner et al, 2011). We therefore hypothesized that MEF2 could actively regulate *p21* transcription by binding to an active enhancer and by recruiting other cofactors, such as the acetyltransferase p300 (Zheng et al, 2013), which in turn cooperate to maintain the open chromatin state. We confirm this hypothesis by performing a ChIP

experiment using the anti-H3K27Ac antibody. Figure 28C illustrates that the highest enrichment for H3K27 acetylation can be observed around position +2.1kb in the same region where MEF2C and MEF2D binding was observed. Furthermore, in cells with down-regulated MEF2D, the acetylation of H3K27 was clearly reduced, specifically at position +2.1kb from TSS.

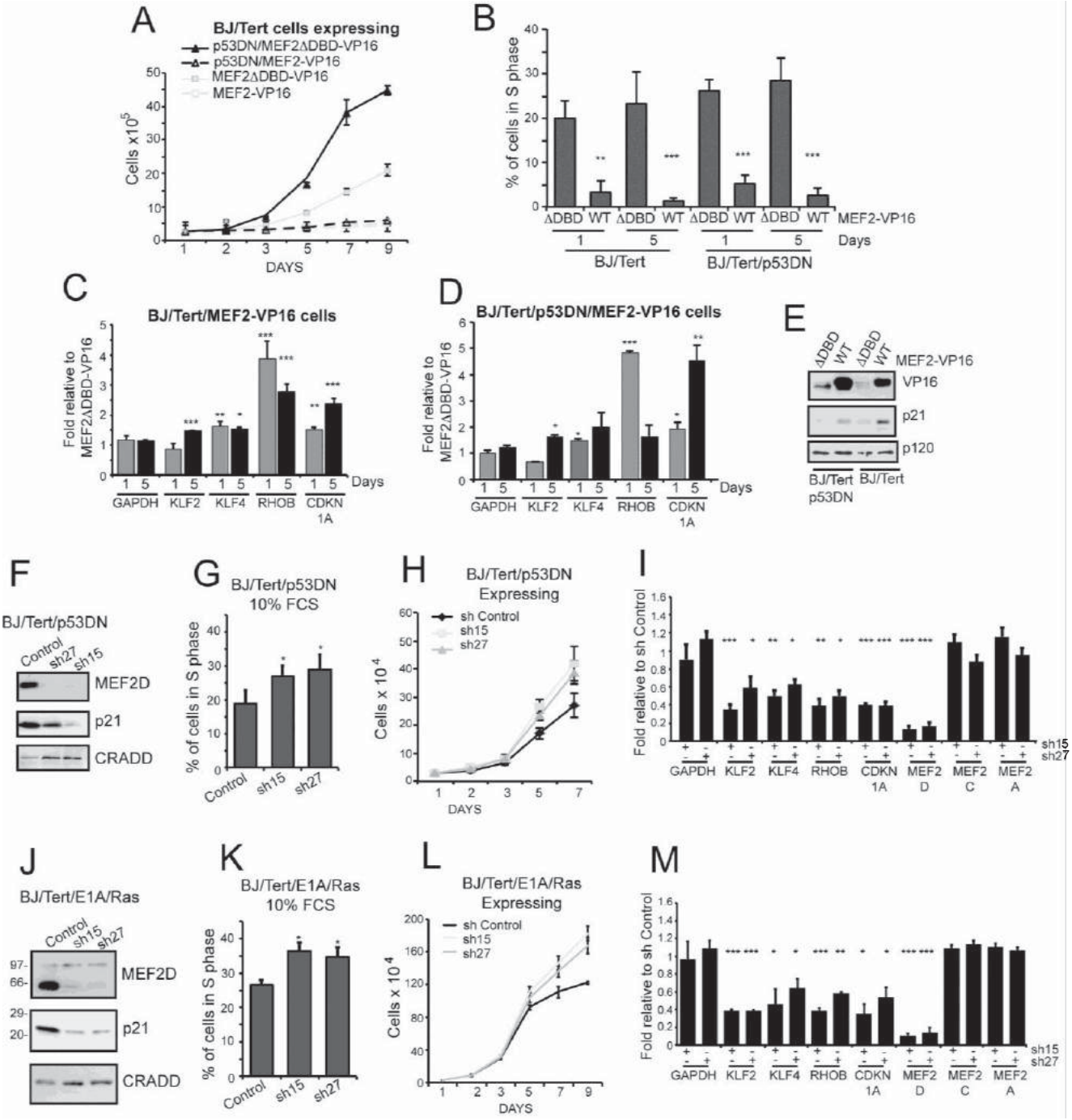


Figure 26 MEF2s affect proliferation of human fibroblasts by inducing CDKN1A expression. (A) Cell-growth assay in BJ/Tert and BJ/Tert/p53DN cells expressing the indicated transgenes and treated with 4-OHT for the indicated times. Data are presented as mean ± SD; n = 3. (B) 1 and 5 days after the seeding BrdU positivity of BJ/Tert and BJ/Tert/p53DN cells expressing the indicated transgenes and treated with 4-OHT for 1 or 5 days was quantified. Data are presented as mean ± SD; n = 4. (C) mRNA expression levels of MEF2-target genes *(KLF2, KLF4, RHOB)* and *CDKN1A* in BJ/Tert cells expressing the indicated transgenes, treated with 4-OHT and collected 1 and 5

days after the seeding. *GAPDH* was used as control. Data are presented as mean ± SD; n = 3. (D) mRNA expression levels of MEF2-target genes *(KLF2, KLF4, RHOB)* and *CDKN1A* in BJ/Tert/p53DN cells expressing the indicated transgenes, treated with 4-OHT and collected 1 and 5 days after the seeding. *GAPDH* was used as control. Data are presented as mean ± SD; n = 3. (E) Immunoblot analysis of p21/CDKN1A in BJ/Tert and BJ/Tert/p53DN cells expressing the indicated transgenes and treated for 60h with 4-OHT. Anti-VP16 and anti-FLAG antibodies were used to reveal the expression of the transgenes. p120 was used as loading control. (F) Immunoblot analysis of p21/CDKN1A in BJ/Tert/p53DN in which MEF2D was knocked-down with two different shRNAs (sh15 and sh27). The efficiency of the down-regulation was proved with an anti-MEF2D antibody. CRADD was used as loading control. (G) Quantification of BrdU positivity of BJ/Tert/p53DN silenced (sh15 and sh27) or not (shCT) for MEF2D. Analyses were performed 48h after seeding. Data are presented as mean ± SD; n = 4. (H) Cell growth assay in BJ/Tert/p53DN cells expressing the indicated shRNAs and grown for the indicated times. Data are presented as mean ± SD; n = 3. (I) mRNA expression levels of MEF2-target genes *(KLF2, KLF4, RHOB)* and of *CDKN1A* in BJ/Tert/p53DN cells in which MEF2D expression was down-regulated using two different shRNA, as indicated. Data are presented as mean fold relative to control (shCT) ± SD; n = 3. (J) Immunoblot analysis of p21/CDKN1A in BJ/Tert/E1A/Ras cells in which MEF2D was knocked-down using two different shRNAs (sh15 and sh27). The efficiency of the down-regulation was proved with an anti-MEF2D antibody. CRADD was used as loading control. (K) Quantification of BrdU positivity of BJ/Tert/E1A/Ras silenced (sh15 and sh27) or not (shCT) for MEF2D. Analyses were performed 48h after the seeding. Data are presented as mean ± SD; n = 4. (L) Cell growth assay in BJ/Tert/E1A/Ras cells expressing the indicated shRNAs and grown for the indicated times. Data are presented as mean ± SD; n = 3. (M) mRNA expression levels of MEF2-target genes *(KLF2, KLF4, RHOB)* and of *CDKN1A* in BJ/Tert/EIA/Ras cells in which MEF2D expression was knocked-down, as indicated. Data are presented as mean fold relative to control (shCT) ± SD; n = 3.

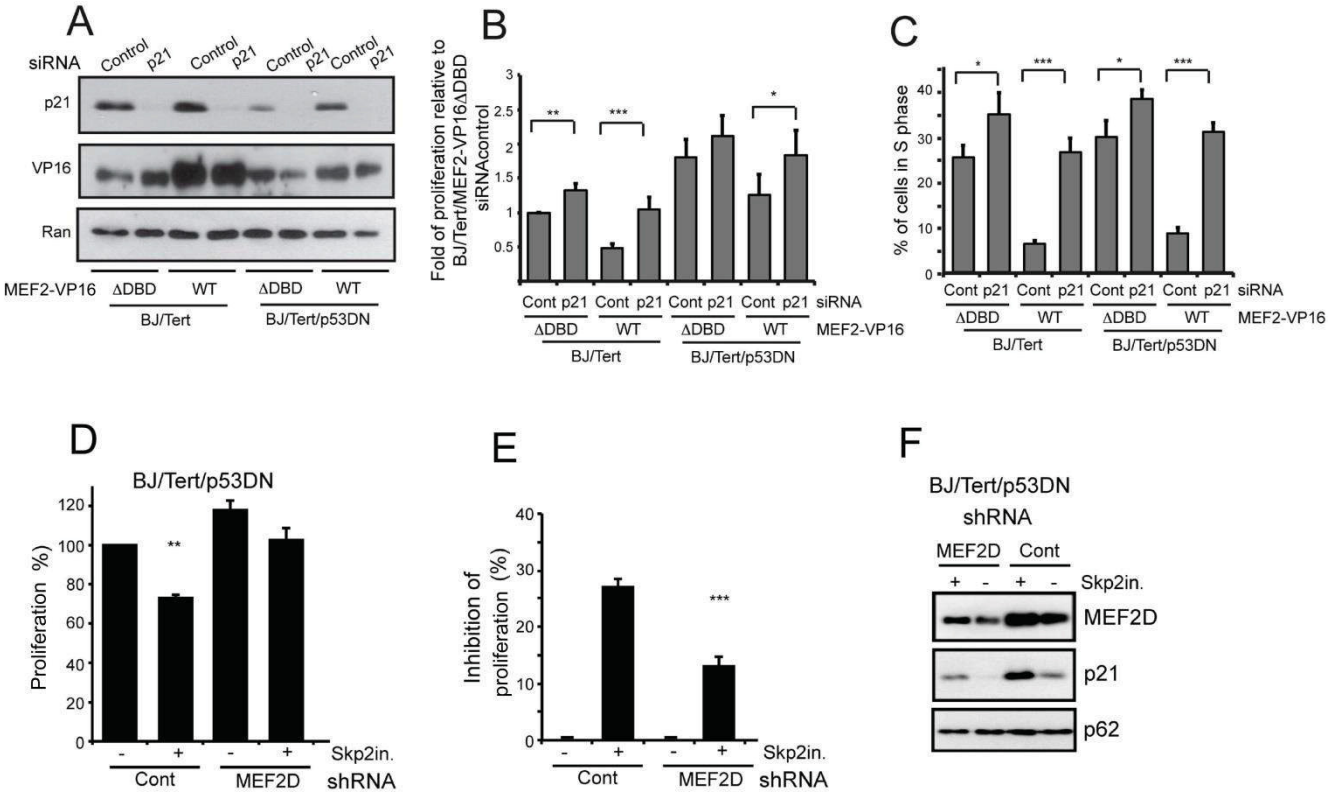


Figure 27 MEF2D anti-proliferative effects relay mainly on its regulation of CDKN1A transcription. (A) Immunoblot analysis of p21 CDKN1A in BJ/Tert and BJ/Tert/p53DN expressing the indicated transgenes, treated with 4-OHT and silenced or not for p21/CDKN1A, as indicated. Anti-VP16 and anti-Ran antibodies were used respectively to reveal the expression of the transgenes and as loading control. (B) Quantification of the proliferation rate of the indicated cell lines relative to BJ/Tert/MEF2ΔDBD cells treated for 36h with siRNA against p21 or siRNA CONTROL. Data are presented as mean ± SD; n = 3. (C) Quantification of BrdU positivity of the indicated cell lines, treated as in B. Data are presented as mean ± SD; n = 4. (D) Quantification of the proliferation rate of BJ/Tert/p53DN cells in which MEF2D was knocked-down or not and treated or not for 24h with the Skp2 inhibitor, as indicated. Data are presented as mean ± SD; n = 3. (E) Percentage of proliferation inhibition in BJ/Tert/p53DN cells knocked-down or not for MEF2D and treated or not with SKP2 inhibitor, as indicated. Data are relative to untreated cells and presented as mean ± SD; n = 3. (F) Immunoblot analysis of MEF2D and p21/CDKN1A levels in BJ/Tert/p53DN cells treated as in 27D. p62 (nucleoporin) was used as loading control.

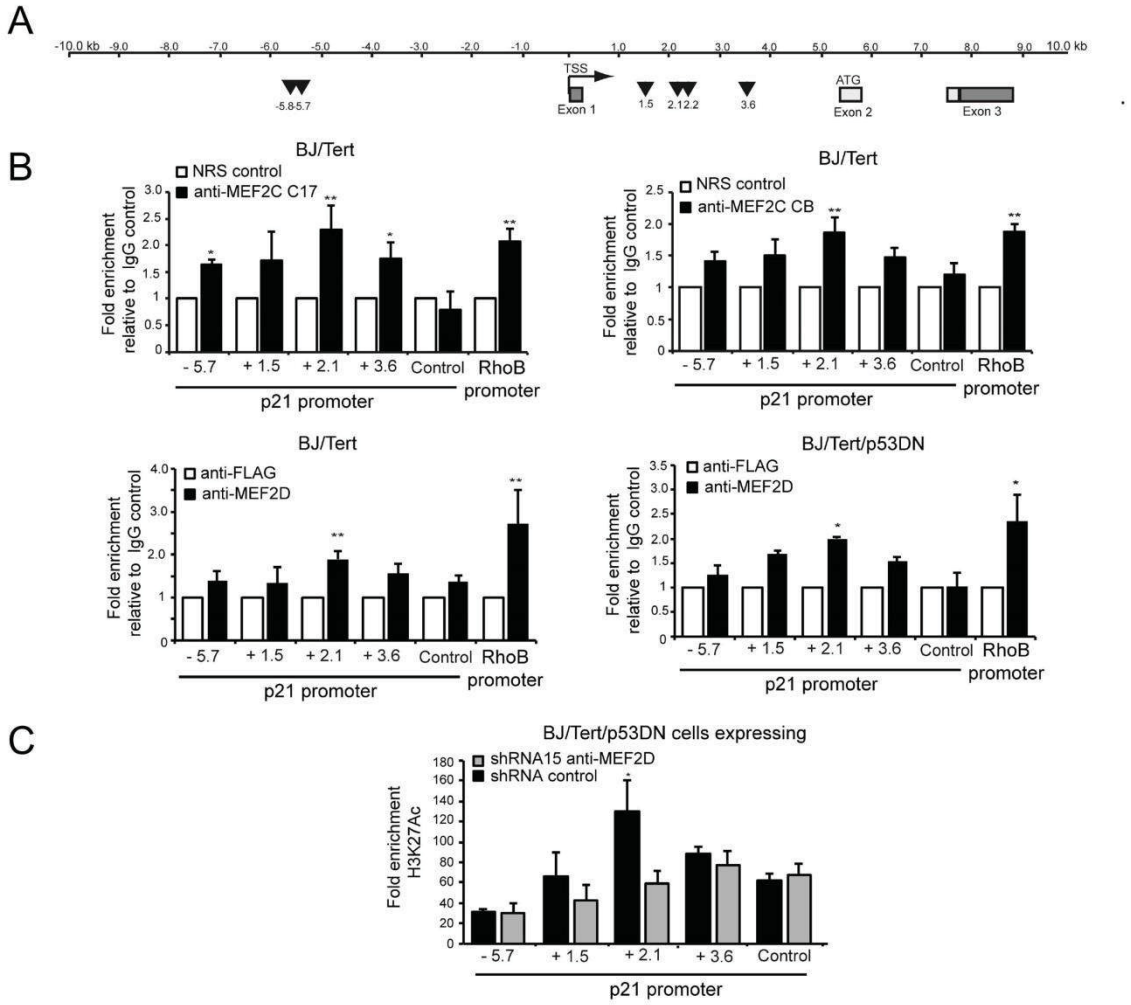


Figure 28 MEF2 directly binds the first intron of *p21/CDKN1A*. (A) Representation of the *p21 CDKN1A* gene structure and its promoter region 10 Kb upstream and 10 Kb downstream from the transcription start site (TSS). The putative MEF2 binding sites are highlighted. The coding (light grey) and the non-coding exons (dark grey) and the ATG leader are also indicated. (B) ChIP on BJ/Tert and BJ/Tert/p53DN cells. Chromatin was immunoprecipitated using two distinct antibodies against MEF2C and one against MEF2D. Normal rabbit serum (NRS) and anti-Flag antibody were used as relative controls. *RHOB* promoter was used as a positive control and an internal region (+4,7 Kb) of the *CDKN1A* gene was used as a negative control (Control). Data are show as fold enrichment relative to the not related control and are presented as mean ± SD; n=3. (C) ChIP of BJ/Tert/p53DN cells in which MEF2D was knockdown with shRNA 15. The H3K27 acetylation status of the putative MEF2 binding sites on *p21 CDKN1A* promoter is shown. Chromatin was immunoprecipitated with antibodies against acetylated H3K27 (H3K27ac) and normal rabbit serum (NRS) was used as relative control. Data are show as fold enrichment relative to the not related control and are presented as mean ± SD; n=3.

The relevance of MEF2-class IIa HDACs axis perturbation in tumours.

In order to investigate the relevance of class IIa HDACs-MEF2 axis *in vivo* in tumours, we adopted a Bioinformatics approach. In particular, we defined a HDAC4-MEF2 signature, constituted of 25 MEF2 targets significantly repressed by HDAC4 in our DNA microarray experiment *(ANXA8, KLF2, SLC36A1, BHLHE41, KLF3, SLC40A1, CDSN, KLF4, SLC7A2, CEACAM1, MSLN , TNFRSF11B, EDN1, NR4A1, TRIB1, FGF7, NR4A2, GADD45B, OXR1, GPNMB, PANK1, HAVCR2, PPAP2A, IRS1, RHOB);* we included in the signature only the validated MEF2-targets. We hypothesized that the tumours in which this signature is represses should be the ones in which the MEF2-HDAC axis is deregulated. Therefore we interrogated the transcriptomes of 14 tumour-types coming from 40 DNA microarray GEO data sets 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 (Figure 29A). In particular, STSs turned out to be the tumours scoring the strongest repression of these 25 genes. The down-regulation of this MEF2 signature in STSs was also confirmed by means of GSEA. The MEF2 signature resulted significantly enriched in normal tissues compared to tumours, and its repression parallels the progression of tumour malignancy (Figure 29B).

Having discovered a correlation between MEF2 transcriptional activity and STSs, we examined the contribution of MEF2 to the tumorigenic phenotype in a panel of human leiomyosarcoma cell lines (LMS). We initially verified whether, similarly to NIH-3T3 and BJ/Tert cells, MEF2D and MEF2C levels were under the control of the proteasome. With the exception of SK-UT-1 cell line, MEF2C and MEF2D protein stability are controlled via the proteasome in LMS cells (Figure 29C). We expected a decreased half-life of MEF2 in cancer cell lines, because of an increased proliferation; this was not true for SK-UT-1 cells, which do not respond in terms of MEF2 stabilization after inhibiting their proliferation with LY294002 (Figure 29C). These cells are characterized by the expression of a variant of SKP2 that is retained in the cytoplasm and fails to direct cyclin D1 ubiquitylation (Ganiatsas et al, 2001). This peculiarity could explain the defect in the poly-ubiquitylation of MEF2 and we plan to confirm this hypothesis by re-expressing the wild-type form of SKP2 in these cells in order to recover the normal turnover of MEF2.

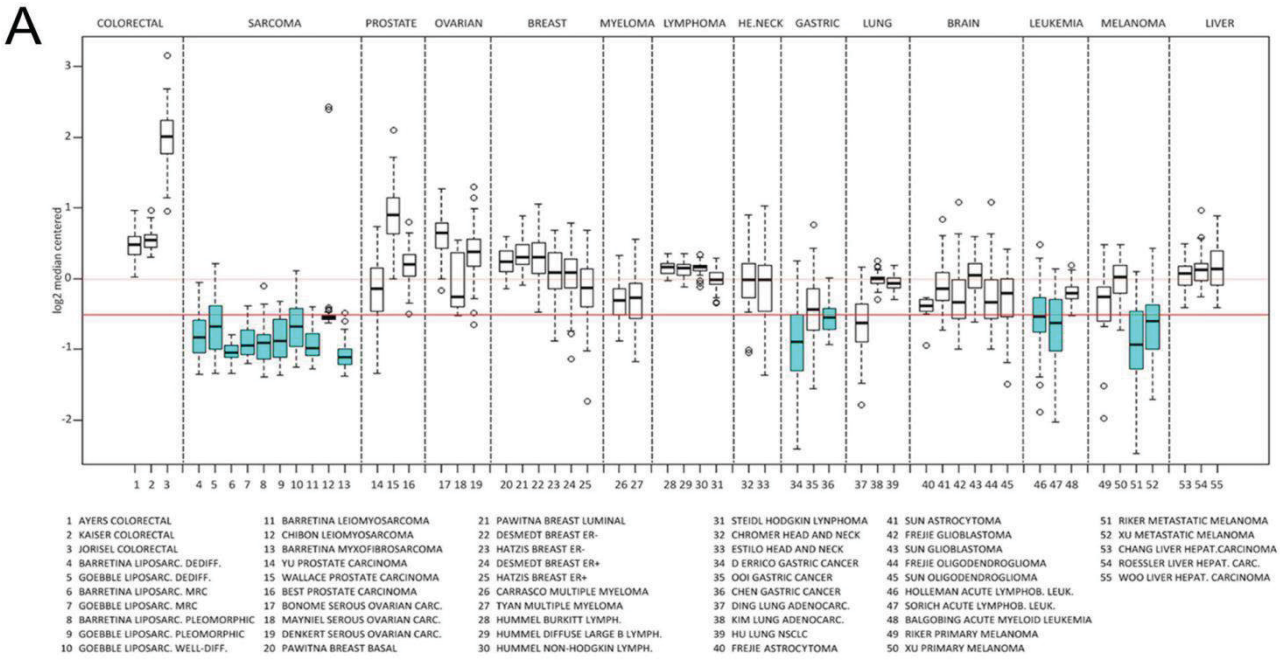
Next, we engineered LMS cells with the MEF2-VP16-ER chimera for inducible MEF2-dependent transcription. The induction of MEF2 was sufficient to reduce the proliferation (Figure 29D), as well as the anchorage-independent growth of LMS cell lines (Figure 29E). These results support the hypothesis of MEF2 as a tumour suppressor in STSs, despite its increased stability in SK-UT-1 cells.

We therefore hypothesized that in these tumours, beside the proteasome-mediated turnover of MEF2 proteins, could act class IIa HDACs. Under this scenario, paradoxically class IIa HDACs and SKP2 could compete for the repression of MEF2. We plan to demonstrate this hypothesis in a dedicated project.

Leiomyosarcomas are in general not responding to traditional chemotherapy and despite an initial responsiveness to PI3K/AKT/mTOR inhibitors, the prolonged treatment resulted in acquired resistance (Hernando et al, 2007; Wong et al, 2014). The urgency of a treatment for these cancers prompted us to firstly demonstrate that the activation of MEF2 signature could be a useful therapy for these tumours. In a pilot study, in order to get MEF2 activation we decided to act in two ways: i) by increasing the stability of MEF2 by means of proliferation inhibitors, such as the pharmacological targeting of the PI3K/Akt pathway; ii) a full MEF2 activation could be obtained with the contemporary inhibition of class IIa HDACs.

We therefore used on LMS cells LY in conjunction with BML-210. This small molecule, although initially identified as a pan HDAC inhibitor, was recently proposed as an inhibitor of the interaction between class IIa HDACs and MEF2s (Jayathilaka et al, 2012). We confirmed that BML-210 discharges the binding between HDAC4 and MEF2D (Figure 30A) and that MEF2 transcriptional activity is augmented in the presence of BML-210 (Figure 30B). In LMS cells both BML-210 and LY inhibited the proliferation of LMS cell lines and, most importantly, the combination of the two drugs shown additive effects in terms of the suppression of proliferation (Figure 30C) and cell-cycle progression (Figure 30D). 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 (Figure 30E). These preliminary results suggest that in this type of

cancer a competition for MEF2 between SKP2 and class IIa HDACs could exist and that in these cancers the activity of class IIa HDACs could be enhanced and required for transforming MEF2 in transcriptional repressors.



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

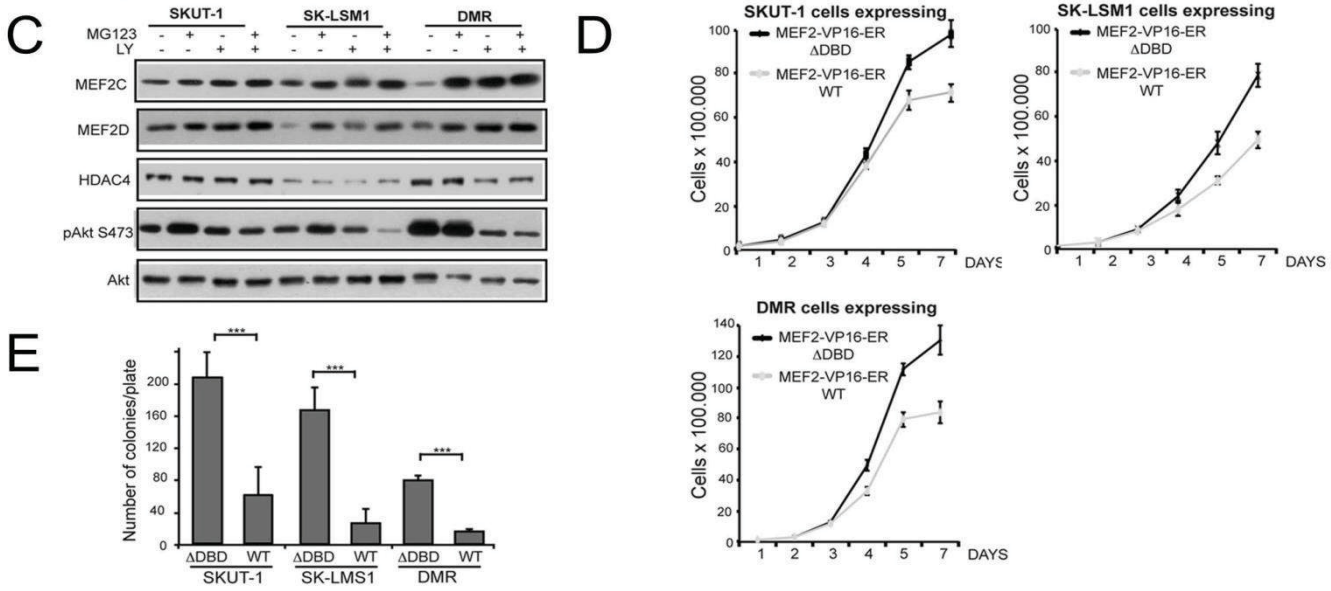


Figure 29 HDAC4/MEF2 signature is repressed in STSs. (A) Bioinformatics analysis results on 40 datasets of tumors interrogated with HDAC4/MEF2 geneset. Box plots depicted in light-blue mark tumors where the MEF2 signature is significantly below the median (zero) and with at least the 50% of the values below an arbitrary threshold of -0.5 (that means that the genes are among the genes less expressed in the array). Significance was calculated by using the Poisson test (Holm-Bonferroni correction, P<0.05). (B) GSEA on STSs,

using the HDAC4/MEF2 signature as a gene set. (C) 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. (D) Cell-growth assay on human sarcoma cells expressing MEF2-VP16-ER or MEF2ΔDBD-VP16-ER grown in the presence of 4-OHT. (E) Quantitative results of colony formation in soft agar of the cells expressing the indicated transgenes. *** $p < 0.001$.

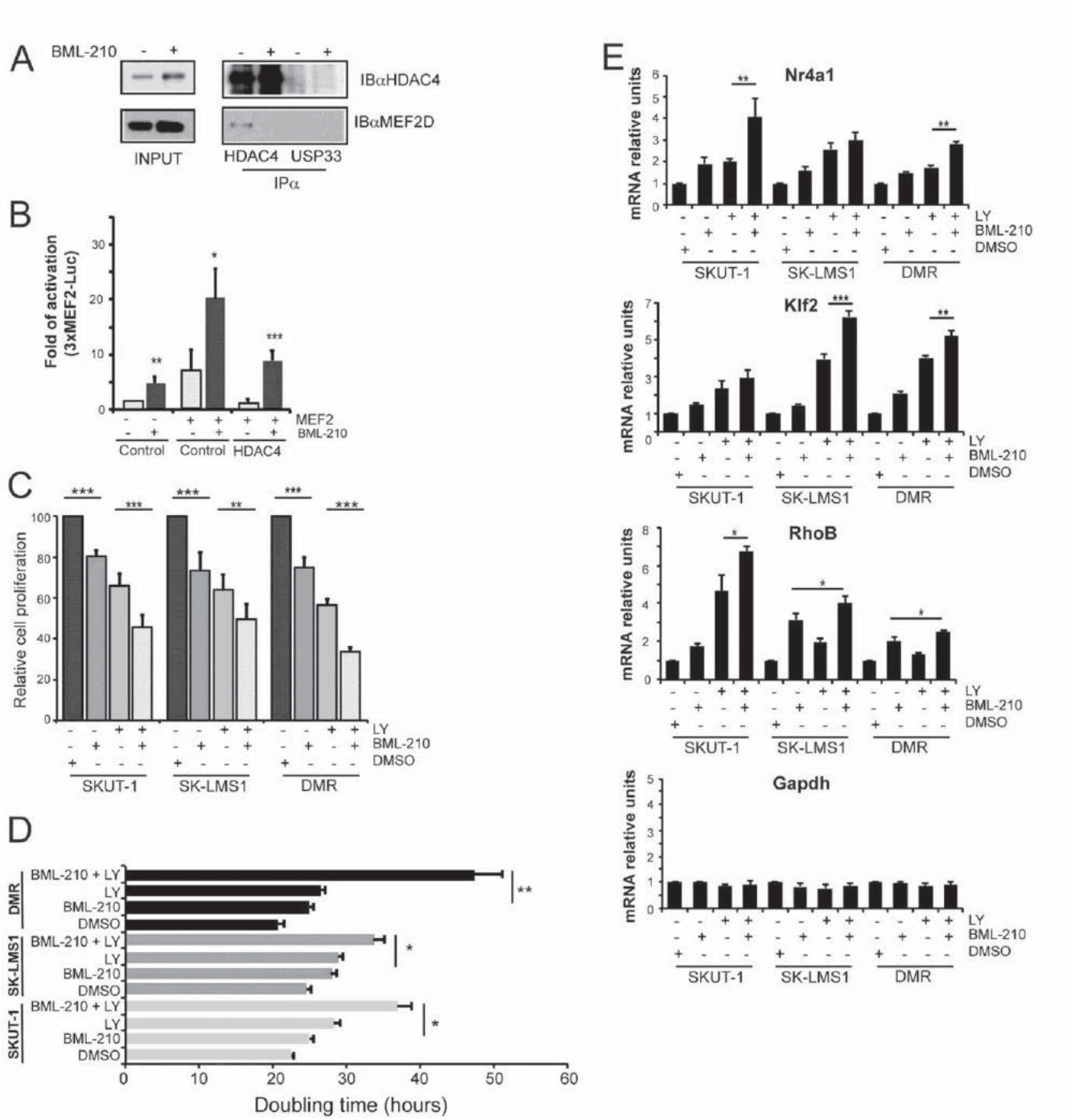


Figure 30 Pharmacological targeting of MEF2-HDAC axis and PI3K/Akt pathway. (A) Co-immunoprecipitation experiment on lysates of IMR90-E1A cells treated or not for 36 h with BML-210 and immunoprecipitated with an anti-HDAC4 antibody. Immunoblots were performed with the anti-MEF2D and anti-HDAC4 antibodies. (B) Luciferase assay on lysates generated by IMR90-E1A cells transfected with the p3xMEF2 luc reporter of MEF2 activity and MEF2C and HDAC4 where indicated and treated or not after 12 h with BML-210 for 36 h. (C) Cell-growth assay on human sarcoma cells 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 C. The DB was calculated according to the

following formula: DB=(t2-t1)*[log2/log(q2/q1)], where t2 is time 2, t1 is time 1, q1 is the number cells at t1, and q2 is the number of cells at t2. (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. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DISCUSSION

Despite more than a decade of studies, the role of class IIa HDACs during the neoplastic transformation is still not completely understood.

Class IIa HDACs oncogenic properties are exerted both in the cytoplasm and in the nucleus. In particular, in the cytoplasm they are involved in Hif-1α stabilization and activation (Kato et al, 2004; Qian et al 2006; Seo et al, 2009), while in the nucleus their main oncogenic property is linked to the down-modulation of p21/CDKN1A got through the repression of Sp1 (Wilson et al, 2008; Mottet et al, 2009), Foxp3 (Liu et al, 2009) or Trsp1 (Wuelling et al, 2014). HDAC7 was reported to increase the expression levels of c-Myc in a panel of cancer cell lines (HeLa, HCT-116 and MCF-7) and its silencing causes a G1/S cell-cycle arrest and the induction of premature senescence (Zhu C et al, 2011); however, literature around HDAC7 is complicated by the fact that in VSMC the cytoplasmic fraction of HDAC7 is involved in blocking the nuclear re-localization and activation of β-catenin (Margariti et al, 2010; Zhou et al, 2011). Based on VSMCs literature it could be possible to attribute to the cytoplasmic fraction of HDAC7 a tumor-suppressor function and to the nuclear fraction a pro-oncogenic property. However a pro-oncogenic property was attributed to class IIa HDACs on the basis of their cytoplasmic activation of Hif-1α (Kato et al, 2004). Moreover, the VSMCs model is not pertinent to colorectal cancer cells, where the nuclear HDAC5 is proposed to be involved in the stimulation of p53 cytostatic properties (Sen et al, 2013). In p53 null-cancer cells it was demonstrated that the knock-down of HDAC4 causes segregation defects of the chromosomes during mitosis and has a strong anti-proliferative effects (Cadot et al, 2009); therefore class IIa HDACs oncogenic properties could be stronger in a p53-null context, where any interference with p53 functions is not possible.

With the attempt to clarify the complex, extensive and sometime controversial literature about the relationships between class IIa HDACs and cancer, we decided to explore the oncogenic potential of a nuclear and of a mainly cytoplasmic form of HDAC4 in not transformed p53 wild-type murine fibroblasts. We demonstrated here that only the nuclear retention of HDAC4 causes the transformation of NIH-3T3 fibroblasts (Figure 12 and 13). This property is largely dependent on a strong repression of MEF2 TFs. In fact, the re-expression of a hyper-active form of MEF2C in the cells transformed by nuclear HDAC4 is sufficient to completely abolish the oncogenic phenotype. Moreover, the expression of a nuclear form of HDAC4 deprived of the ability to repress MEF2 has no oncogenic properties (Figure 15).

With our study we therefore proposed that the real discriminant for class IIa HDACs oncogenic roles are the MEF2s. The MEF2 TFs are the stronger partners of class IIa HDACs (Miska et al, 1999), however the behavior of class IIa HDACs-MEF2 axis in cancer cells has been poorly explored and most of the reported oncogenic/tumor suppressor qualities of class IIa HDACs are independent from MEF2s regulation. In our lab it was for the first time investigated the role of HDAC-MEF2 axis in cancer. We demonstrated that the alteration of the HDAC-MEF2 axis could result in more aggressive ER+ breast cancers (Clocchiatti et al, 2013) and it was sufficient for the acquisition of hallmarks of transformation by normal fibroblasts (Di Giorgio et al, 2013).

We are persuaded that the repression of MEF2 results in the acquisition of a proliferative advantage. In particular we have demonstrated that for exerting their proliferative functions several oncogenes (RAS and AKT) requires the repression of MEF2 TFs (Figure 19). Moreover, mitogen stimulation of cell-growth causes a

transient stimulation of MEF2 activities in normal fibroblasts cells, as reported previously by others (Han and Prywes, 1995; Suzuki et al, 1995; Kato et al, 1997); however, this stimulation is quickly exhausted and come out in a repression of MEF2 transcriptional activity (Figure 20). We associated this decrease in MEF2 activities to the proteasomal/SKP2-mediated degradation of MEF2C and MEF2D in response to a phosphorylation-dependent signal conveyed by the CDK4/CyclinD1 complex (Figures from 20 to 23). For the first time we described a cell-cycle controlled regulation of MEF2 activities; this degradation of MEF2C and MEF2D is required by normal fibroblasts for entering the S phase of the cell-cycle (Figure 24 and 26). A large fraction of the MEF2 TFs anti-proliferative signal is due to their direct transcription of p21 CDKN1A (Figure 25 and 27), that requires the binding of MEF2 to the first intron of p21 and the induction of H3K27 acetylation (Figure 28).
We found that STS sarcomas are tumors largely dependent on MEF2 repression exerted by class IIa HDACs (Figure 29 and 30). Preliminary data, based on the comparison of the effects between HDAC4 over-expression and MEF2D knock-down, suggest that this mechanism of MEF2 inactivation seems to be stronger in respect to their proteasomal degradation (Figure 29, 30, 31 and data not shown); it is possible that the binding of class IIa HDACs to MEF2 could transform these transcription factors in transcriptional repressors that act as dominant effectors counteracting the positive influence of other TFs acting on the same promoter/enhancer. In other words, MEF2 could remain bound on the regulative regions/promoters of anti-proliferative genes (such as for example *p21, RHOB, KLF2, GADD45B*), and keeping their transcription off. In particular, a cell-cycle dependent regulation of MEF2 activities could be exerted in normal cells and pre-malignant lesions at the protein level, while the considerable up-regulation of HDAC4 with the increase of malignancy (preliminary data not shown) could offer to cancer cells a stronger way to abolish the transcription of MEF2 targets. Alternatively, as suggested by the additive anti-proliferative effects obtained by the combined targeting of PI3K/AKT pathway and of the MEF2/HDAC axis in sarcoma cells (Figure 31), a cooperative mechanism between class IIa HDACs and SKP2 is also conceivable. These extremely interesting aspects should be investigated better in the next future.

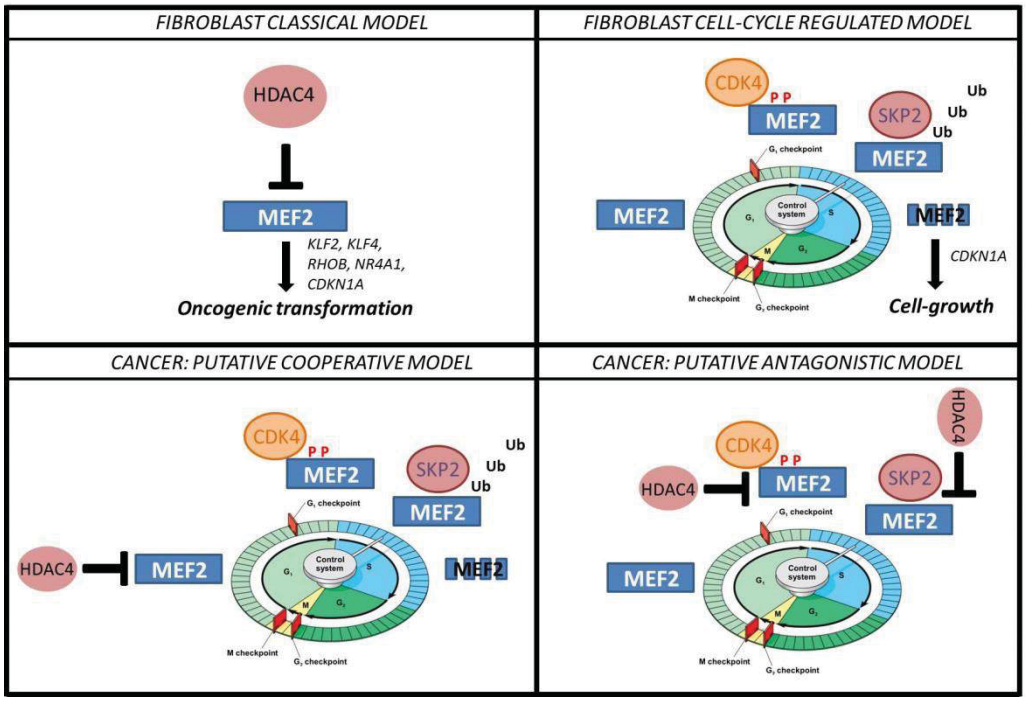


Figure 31 MEF2 is the converging hub of two different pathways that mediate its repression in normal fibroblasts. Are these two models antagonistic or cooperative in cancer cells?

However, also in this case the literature about MEF2 involvement in oncogenesis is controversial, and these transcription factors are considered strong tumor suppressors in sarcomas (Di Giorgio et al, 2013; Zhang M et al, 2013; Zhang et al, 2014), but mild oncogenes strongly up-regulated in blood cancers (Cantè-Barret et al, 2013) and in hepatocarcinomas (Ma et al, 2014). Several publications attribute to MEF2 a transforming potential in these latter two contexts, however when the proliferation of the cancer cells after the knock-down of a MEF2 was scored no effects (Schwieger et al, 2009) or an increase in the proliferation of the cells was reported (Bai et al, 2014). The common message of all these papers is that MEF2 TFs increase the invasiveness of cancer cells, in the blood compartment by enhancing the stemness qualities of the malignant cells (Krivtsov et al, 2006; Schwieger et al, 2009; Homminga et al, 2011), in hepatocarcinomas by inducing the EMT transition (Yu et al, 2014) and vasculogenesis (Bai et al, 2014). Moreover MEF2 regulates the transcription of a plethora of inflammatory genes, such as the ones belonging to MMP and KLF family (Han et al, 1997; Palmar et al, 2006), which could positively or negatively influence oncogenesis, according to the strength and the timing of the response (Coussens et al, 2013).

How reconcile sarcoma, hepatocarcinoma and blood cancer models? I speculate that MEF2 tumor suppressor roles could be prevalent in mesenchymal tumors, where any role of MEF2 as EMT promoter is unnecessary. In particular I suppose that for the expansion of the malignant cells it is a necessary and sufficient condition having the MEF2 signature off. According to the district in which the tumor arises, MEF2 reactivation could be then necessary for the acquisition of some invasive features (e.g. in hepatocarcinoma EMT, in other cancers inflammation) and again switched-off during the subsequent proliferative phase. A panel of focused *in vivo* studies in murine models of carcinogenesis in which the activity of MEF2 TFs could be measured in real time, by means of β-galactosidase reporter assays or better through ChIP-seq experiments coupled to DNA microarrays, could confirm or reject this hypothesis.

Many authors have hypothesized that MEF2s bind to the genome in a context-dependent way. The first experiments of ChIP-seq demonstrate that this hypothesis holds true (Johnson et al, 2014; Wales et al, 2015); this is not surprising and would include MEF2 in a very big family of TFs with a context-dependent activity (Wang K et al, 2009). However, some key aspect, such as the MEF2-dependent regulation of *p21* transcription should be a common feature in many districts.

Given these premises and the opposing roles of MEF2 in many cancers and probably during the progression of the cancer itself, would it be possible to consider MEF2 pathway for a therapeutic approach? The answer to this question implies before the answering to another question: do MEF2 repressors, such as class IIa HDACs and Cabin1, when active, bind MEF2 proteins and repress their activity in all the genomic regions bound by the transcription factors or do they act preferentially on some loci? In order to answer to this question we plan to perform a ChIP-seq experiment to map all the genomic regions bounded by HDAC4 at different levels of transformation (BJ, BJ/E1A, BJ/E1A/RAS, BJ/E1A/RAS/p53DN) and determine the dependence of this binding from MEF2 through a re-ChIP approach. This will allow clarifying if some oncogenic stimuli are sufficient to convey to class IIa HDACs a certain degree of specificity in their repressive activity.

Another open question concerns the pool of genes induced by HDAC4; among them many have strong oncogenic properties *(HGF, MERTK, GLI2)*. The study of their regulation is less intuitive as their up-regulation induced by HDAC4 implies the repression of a repressor. Its identification should open an extremely interesting field of study.

In summary, our work remarks the oncogenic potential of class IIa HDACs and underlines the absolute necessity of the development of specific drugs capable of abolishing their oncogenic activity. We demonstrate that MEF2 TFs behaves as strong negative regulators of fibroblasts and sarcoma cells proliferation and that cell-cycle progression requires their degradation or the repression by class IIa HDACs. The latter seems to be the stronger.

Finally, we are persuaded that molecules similar to BML-210 designed to displace class IIa HDACs from MEF2 could represent promising therapeutic avenues for the treatment of soft-tissue sarcomas, in particular for those tumors, such as leiomyosarcoma, that are still orphan of a valid therapeutic approach.

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In the meanwhile in Italy everyday about a thousand people receive a new diagnosis of cancer; the fifthy percent of them will die because of cancer in the following five years.
In every single moment of my experience in the lab I thought to those people and their families and I tried to contribute to the best of my capabilities in this fight against this terrible disease.
Therefore, every my scientific result will be first of all always dedicated to people with cancer; my hope is that one day, maybe by the brilliant idea of a student, a cure, perhaps universal, could be found.

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I wish to conclude with a promise (from “Not Again”, by Linda Nielsen, talking about her fight against cancer)

“Sometimes it happens in a race,
a false start or two we’ll see.
But to keep on running hard and strong,
brings only victory.

Yes, it really makes me angry,
that another fights begun.
But all along I’ve always known,
that this one WILL be won!”

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APPENDIX

List of publications during the PhD:

1: Di Giorgio E, Gagliostro E, Brancolini C. **Selective class IIa HDAC inhibitors: myth or reality.** Cell Mol Life Sci. 2015 Jan;72(1):73-86. doi: 10.1007/s00018-014-1727-8. Epub 2014 Sep 5. PubMed PMID: 25189628.

2: Di Giorgio E, Clocchiatti A, Piccinin S, Sgorbissa A, Viviani G, Peruzzo P, Romeo S, Rossi S, Dei Tos AP, Maestro R, Brancolini C. **MEF2 is a converging hub for histone deacetylase 4 and phosphatidylinositol 3-kinase/Akt-induced transformation.** Mol Cell Biol. 2013 Nov;33(22):4473-91. doi: 10.1128/MCB.01050-13. Epub 2013 Sep 16. PubMed PMID: 24043307; PubMed Central PMCID: PMC3838174.

3: Clocchiatti A, Di Giorgio E, Ingrao S, Meyer-Almes FJ, Tripodo C, Brancolini C. **Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of ER$^+$ breast tumors.** FASEB J. 2013 Mar;27(3):942-54. doi: 10.1096/fj.12-209346. Epub 2012 Nov 16. PubMed PMID: 23159930.

4: Clocchiatti A, Di Giorgio E, Demarchi F, Brancolini C. **Beside the MEF2 axis: unconventional functions of HDAC4.** Cell Signal. 2013 Jan;25(1):269-76. doi: 10.1016/j.cellsig.2012.10.002. Epub 2012 Oct 11. Review. PubMed PMID: 23063464.

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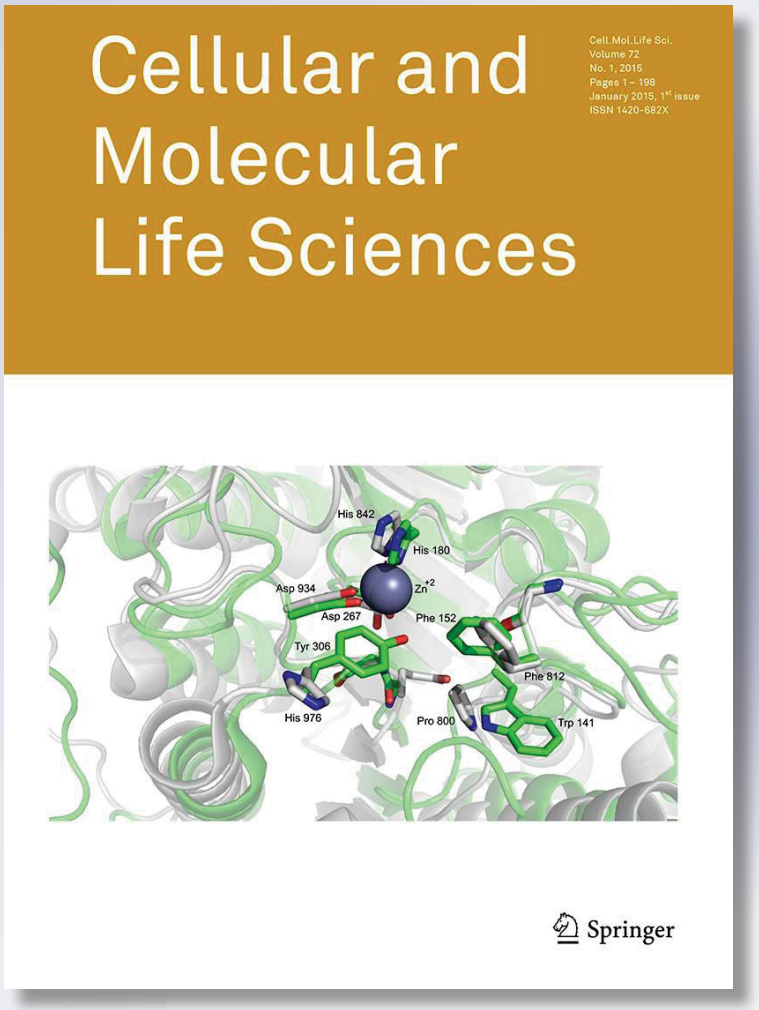

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RESEARCH ARTICLE

Selective class IIa HDAC inhibitors: myth or reality

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Abstract The prospect of intervening, through the use of a specific molecule, with a cellular alteration responsible for a disease, is a fundamental ambition of biomedical science. Epigenetic-based therapies appear as a remarkable opportunity to impact on several disorders, including cancer. Many efforts have been made to develop small molecules acting as inhibitors of histone deacetylases (HDACs). These enzymes are key targets to reset altered genetic programs and thus to restore normal cellular activities, including drug responsiveness. Several classes of HDAC inhibitors (HDACis) have been generated, characterized and, in certain cases, approved for the use in clinic. A new frontier is the generation of subtype-specific inhibitors, to increase selectivity and to manage general toxicity. Here we will discuss about a set of molecules, which can interfere with the activity of a specific subclass of HDACs: the class IIa.

Keywords SAHA · HDAC3 · HDAC4 · HDAC5 · HDAC7 · HDAC9 · MEF2 · p21 · Therapy · Apoptosis · Cell cycle · Anti-cancer · Neurodegeneration · Inflammation

Introduction

Why to target HDACs?

Every complex cellular adaptation and behavior is supervised by changes in the transcriptional machinery, which align the gene expression profile of a specific cell type to the general requirements of the organism. The harmonic regulation of genes transcribed in a specific instant is the result of an integrated and complex network of signals that controls the activity of different transcriptional players. Transcription factors (TFs), epigenetic regulators and "structural" proteins, constituting the chromatin are the chief protagonists under the tight influence of the environment. Alterations in the signaling networks or in the transcriptional players are responsible for aberrations in tissue homeostasis and triggering events in several different diseases, from neurodegeneration up to cancer [1, 2]. The opportunity to reset the transcriptional subverted context, with the therapeutic perspective of curing/alleviating diseases, straightway attracted scientist's attention [3, 4].

Perhaps the simplest approach to develop new drugs is the identification of small molecules, acting as inhibitors of an enzymatic activity that is imperative in a specific disease. In the context of gene transcription, post-translational modifications (PTMs) of histones represent realistic targets for the development of epigenetic therapies aimed to amend transcriptional alterations. Acetylation of lysines, placed in histones but also in TFs is an important PTM, exerting both positive (H3K4, 9, 14, 17, 23; H4K5, 8, 12, 16) and negative (in the case of specific TFs) effects on gene expression [5, 6]. Being acetylation reversible and under the scrutiny of different family of enzymes: HATs (histone acetyl transferases) and HDACs (histone deacetylases), it has attracted several interests as a druggable PTM [7]. In particular, during the past decades, many efforts have been made to isolate, synthesize and characterize small molecules targeting HDACs [8]. HDACis are nowadays represented as a considerable fraction of the epigenetic drugs under study and in some circumstances

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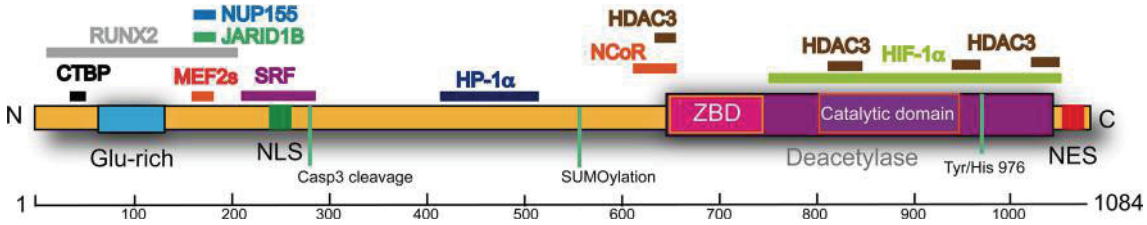


Fig. 1 Schematic representation of class IIa HDACs highlighting the principal domains. As prototype of class IIa we selected HDAC4. Certain interaction partners, as well as the relative HDAC4 sequences involved, are illustrated

these compounds have been approved for the use in clinic (see below). Importantly, epigenetic drugs in cancer therapy represent an opportunity to revert drug-resistance-associated epigenomes and to prevent or reverse non-responsiveness to anti-cancer drugs [2].

Copious studies on cancer cells' epigenomes have fully justified the rationale of applying HDACis in anti-cancer therapies. Three major intrinsic features of the neoplastic cells could be subject of specific intervention, thanks to HDACis: (1) cancer cells are characterized by an enhanced degree of heterochromatinization compared to normal cells; which makes cancer genomes inaccessible to DNA-damage response enzymes [9]. The treatment of cancer cells with HDACis relaxes chromatin and allows the activation of the DNA-damage response [9]. (2) Several tumor suppressor genes, including some pro-apoptotic genes, are inactivated in cancer cells because of ipo-acetylated promoters [3, 10, 11]. (3) Alterations of the epigenetic machineries embracing HDACs are frequently observed in tumors [12, 13].

Despite the considerable literature debating the use in epigenetic therapies of pan-HDACi and of class I HDACs specific inhibitors [10, 11, 14–22], reviews specifically discussing of molecules acting as inhibitors of class IIa HDACs, are quite rare. In this manuscript we will discuss specifically of them.

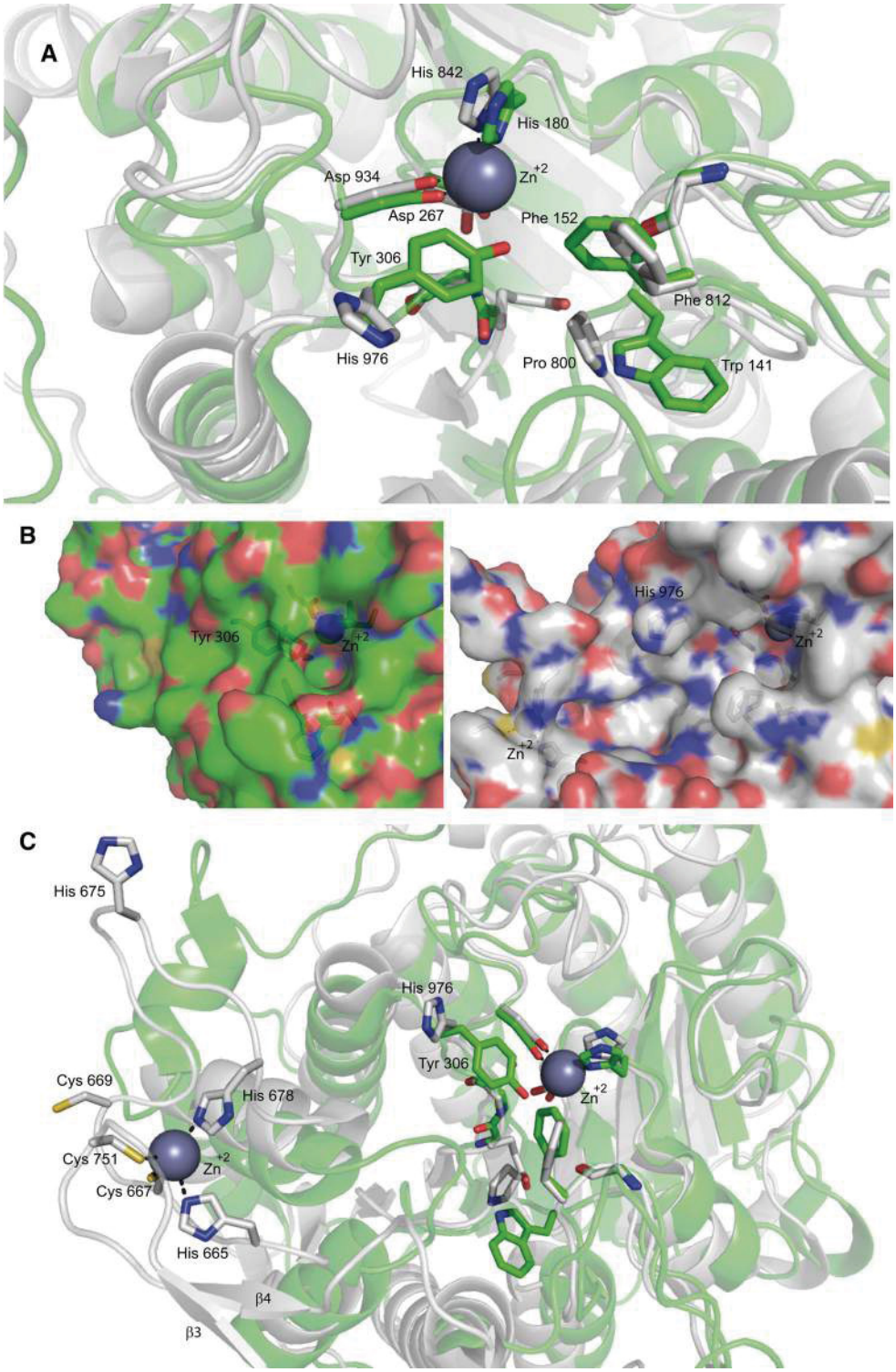
Class IIa HDACs: to be or not to be a lysine deacetylase

In humans there are 18 HDACs grouped into five different classes according to phylogenesis and sequence homology [7]. Class I HDACs (including HDAC1, 2, 3 and 8), class IIb HDACs (including HDAC6 and 10), class III HDACs or Sirtuins (including all Sirtuins from 1 to 7) and class IV (HDAC11) all displaying enzymatic activities [23]. By contrast, when we discuss about class IIa HDACs (HDAC4, 5, 7 and 9) as histone deacetylases, it should be taken into account that these proteins show an extremely low enzymatic activity against acetylated lysines [24, 25] and are rarely associated with histone tails [26].

Structurally, class IIa HDACs can be divided into two parts: the N- and the C-terminal regions (Fig. 1). The N-terminal regulates the nuclear import and contains a coiled-coil glutamine-rich domain that is peculiar of the family. This region is highly devoted to protein–protein interactions both in terms of homo- and of heterotypic partners. The C-terminal region contains the catalytic "deacetylase" domain and the nuclear export sequence (Fig. 1). These enzymes are under the control of different signaling pathways, which operate through specific PTMs to influence peculiar aspects of the class IIa biology, including the nuclear/cytoplasmic shuttling (for reviews [7, 12, 27, 28]).

The deacetylase domain is made up of approximately 400 residues (aa) arranged into 21 α-helices and 10 β-strands organized in a single domain, structured around a central "catalytic" Zn^{2+} ion [29]. Likewise to class I HDACs, 2 aspartates and an histidine coordinate this Zn^{2+} while 2 other aspartates (Fig. 2a), another histidine, a serine and a leucine coordinate two potassium ions [29–31]. Despite this high similarity, in vertebrates class IIa possess a bigger active site than class I HDACs (Fig. 2b), which impacts on their druggability [26, 29]. The evolution-related event responsible for this structural peculiarity is the mutation of a tyrosine into a histidine, Y967H in HDAC4 [25]. Histidine is sterically less cumbersome and induces the relaxation of the structure. As a consequence, this histidine is far from the central Zn^{2+} and not able to form hydrogen bonds with the intermediate of the enzymatic reaction (Fig. 2a). The intermediate is, therefore, very unstable, thus resulting in an ineffective reaction. Nevertheless, class IIa can efficiently process alternative substrates such as trifluoroacetyl-lysine. Mechanistically, the presence of the trifluoro group should destabilize the amide bond, hence favoring the reaction even in the absence of transition-state stabilization [25].

Importantly, replacing back the His with Tyr generates class IIa HDACs with a catalytic efficiency 1,000-fold higher compared to the wild-type (wt) form [25, 31]. Nonetheless, this mutant does not show enhanced repression respect to the wt, at least in the instance of MEF2-dependent transcription, a well-known class IIa partner [25].



Another distinctive feature of class IIa HDAC catalytic site is the existence of a Zinc Binding Domain (ZBD). This ZBD consists in a β-hairpin surrounded by two antiparallel β-strands, forming a pocket-like structure that accommodates a second "structural" zinc ion [29]. In the case of HDAC4 three cysteines (667, 669, 751) and one histidine

◀**Fig. 2** Representation of class I and class IIa catalytic sites (**a**, **b**) and the zinc binding domain (**c**). **a** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic sites. As mentioned in the text the His 976 is rotated away from the active site differently from Tyr 308 in HDAC8. **b** Surface representation of class I HDAC8 (*green*) and class IIa HDAC4 (*white*) catalytic sites. The figure shows the hydrophilic tunnel necessary for the release of the reaction product in HDAC8 (*green*), while in HDAC4 (*white*) the His/Tyr substitution prevents tunnel formation. **c** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic site (*right*) and zinc binding domain of HDAC4 (*left*). β3 and β4 are the two antiparallel β-strands involved in the formation of the pocket-like structure in the zinc binding domain. Importantly, His 665 and His 678 in this inhibitor-bound structure are replaced by Cys 669 and His 675 in the coordination of the zinc ion in the Apo-structure. Unfortunately the crystallization of Apo-HDAC4 was unsuccessful and these differences are deduced from crystallographic studies of the mutant GOF (H976Y) of HDAC4 [31]. The coordinates of the protein structures were retrieved from the protein data bank. Amino acids discussed in the text are labeled and shown in stick representation. The accession codes for the protein structures are: 2VQJ (HDAC4) and 1T69 (HDAC8). Figures are edited using PyMOL Molecular graphics system, Schrödinger, LLC

(675), conserved only among class IIa HDACs, coordinate this Zn^{2+} and made the so-called "core" of the domain [31] (Fig. 2c). Importantly, the inhibitor-bound structure is shown in this figure, where, respect to the Apo-structure, Cys 669 and His 675 replace His 665 and His 678 in the coordination of the Zn^{2+}.

This domain is extremely flexible and the oxidation of the cysteines involved in Zn^{2+} coordination (667 and 669 in HDAC4) is sufficient to free the metal, with the consequent opening and deconstruction of the ZBD [31]. Because this domain is head-to-head to the active site (Fig. 2c), it contributes to make the class IIa HDACs' catalytic site more accessible than that of class I HDACs (Fig. 2b) and does not allow the formation of an efficient hydrophilic tunnel necessary for the release of the acetate reaction product [30, 31].

Old structures and new functions

The enzymatic ineptitude of vertebrates' class IIa deacetylase domain raises several questions and opens the door to different hypothesis. First, they are not completely silenced enzymes. Because class IIa is capable of processing trifluoroacetyl-lysine with high efficiency, still undiscovered new natural substrates could exist [25]. Alternatively, the described enzymatic activity could simply mark a lab finding, without biological implications. Second, as anticipated above, the absence of improved repressive influence in the case of the gain of function His/Tyr substitution in HDAC4, further demonstrates that class IIa HDACs can repress transcription independently from the deacetylase domain [25]. The relevance of the deacetylase-independent repression is testified by MITR, a splice variant of HDAC9 lacking the deacetylase domain [32]. The existence of MITR supports the possibility that the HDAC domain is of little relevance for the functions of class IIa HDACs and may lead to believe that it is an evolutionary heritage intended to being missed. However, since class IIa deacetylase domain has been preserved behind two duplication events occurred during evolution of vertebrates, evolutionists deny the hypothesis that this domain would be subjected to a negative purifying selection [33].

Although there are evidences pointing to deacetylase-independent activities of class IIa, generation of a mouse model in which, mutated versions of this domain can be analyzed in a physiological context will help our understanding. This point is of crucial relevance for the design and development of class IIa inhibitors.

Along with the enzymatic activity, the deacetylase domain can operate as a scaffold for the recruitment of multi-protein complexes containing class I HDAC3 and other co-repressors [31]. HDAC4 interacts with the RD3 domain of N-CoR [24, 34], while HDAC3 binds the SAINT domain [35] and, as a matter of fact, HDAC4 binds N-CoR/SMRT regardless of HDAC3 and only in a second time the deacetylase is recruited [36]. However, the precise order of the sequential molecular interactions driving the assembly of the multi-protein complex is still waiting for a final verification.

When class IIa HDACs are isolated under native conditions, a lysine deacetylase activity can be measured. This activity is due to class I HDACs co-purified with class IIa [24, 37, 38]. The existence of a heterogeneous repressive complex complicates the assessment of effectiveness and specificity of HDACis, when tested on proteins purified from cells or tissues.

A final consideration refers to a fascinating hypothesis, which attributes to class IIa deacetylase domain the function of acetylated lysine reader [26]. In this view, class IIa could act as readers and interpreters of the histone code, thus orchestrating the epigenetic status thanks to their capability of recruiting additional enzymes, such as methylases [39] or deacetylases [24, 36]. A scenario where class IIa HDACs, acting as molecular scaffolds supervise the introduction of different epigenetic markers, onto specific regions of chromatin or in proximity of different acetylated cellular protein. In this context inhibitors of the deacetylase domain could in principle both interfere with the reading activity or, by promoting structural changes, with the possibility of recruiting additional co-repressors.

Unresolved issues

Biochemically, the enzymatic activity associated to class IIa HDACs could be explained by the recruitment of class I enzymes [24]. Moreover, all the point mutants of the HDAC4 deacetylase domain which, accordingly to Finnin model [40], abrogate its enzymatic activity (H803A, G811A, D838A, D840A, H842A, N845D, D934 N, E973G) demonstrate a perfect correlation between enzymatic activity and the ability to recruit HDAC3 [24]. Classic deacetylase activity is not associated with a cytoplasmic HDAC7 or HDAC4 immunoprecipitated from HEK293 cells and therefore, weakly associated to the mainly nuclear HDAC3 [24, 36]. Similarly, HDAC4 mutants that have lost the ability of binding to N-CoR/SMRT drop the deacetylase activity [24]. Despite in vitro binding experiments prove that the fraction of HDAC3 in complex with HDAC4 is relevant, in vivo HDAC3 preferentially forms homodimers, rather than heterodimers with HDAC4 [41]. Furthermore, the fraction of HDAC4 co-purified with HDAC3 in mammalian cells is extremely low [24, 35, 37].

As aforementioned, another peculiar feature of class IIa deacetylase domain is its sensitivity to redox conditions [31, 42]. Particularly, in HDAC4 the oxidation of cysteines 667 and 669 induces the formation of a disulphide bond that causes the exposition of the NES, the export in the cytoplasm and also the detachment of HDAC3 [31, 42, 43]. This oxidation causes the de-structuration of the HDAC domain because Cys 667 and Cys 669 are directly involved in the "structural" Zn^{2+} coordination and substrate binding [29, 31] (Fig. 2c). These findings show that researchers should be extremely cautious in verifying the redox status when studying class IIa deacetylase domain.

In addition to nuclear roles of class IIa HDACs, recently, a cytoplasmic enzymatic activity has been reported towards non-histone substrates [reviewed in 44]. During muscle denervation HDAC4, which plays a pro-atrophic role in this context [45, 46] can deacetylate and activate MEKK2 [47]. Kinase engagement culminates in AP-1 activation and cytokines production that stimulate muscle remodeling [47]. Interestingly only the wild-type form, capable of shuttling between the nucleus and the cytoplasm and not a nuclear resident mutant of HDAC4 deacetylated MEKK2. Importantly, this activity is independent from HDAC3 and is not shared with HDAC5 [47]. Paradoxically, MEKK2 activation should activate ERK5 and therefore MEF2s, thus pointing to a positive rather than repressive influence of HDAC4 versus MEF2s [48, 49]. A similar cytoplasmic KDAC (lysine deacetylase) activity of class IIa HDACs was reported towards HIF-1α and STAT-1. Also in these circumstances class IIa deacetylase activity seems to be independent from class I HDACs [44].

Another unresolved issue is the requirement of additional factors to exert the full enzymatic activity. Class I HDACs require particular cofactors both for histone and non-histone substrates [35, 41, 50]. For the enzymatic activity of class IIa HDACs towards the synthetic trifluoroacetyl-lysine or against these cytoplasmic partners, any cofactor seems to be dispensable [25].

The rationale for developing class IIa HDACs inhibitors

HDACis have entered multiple clinical trials principally in virtue of their anti-neoplastic properties [10]. Much more emphasis has been pushed on the identification, synthesis and characterization of class I HDACis. Commonly HDACis show a selective cytotoxicity against tumor cells and weak effects on normal ones [11, 51, 52]. These molecules display cytostatic effects, especially through the induction of p21 and blockage of the cell cycle [53, 54] or by triggering apoptosis via multiple mechanisms [11, 53, 55, 56]. Some HDACis in vivo stimulate also the clearance of tumor cells from the immune system [57, 58] or block angiogenesis [59, 60]. Despite these promising anti-neoplastic properties, entering of HDACis in clinic is slower than expected, principally due to some side effects and toxicity displayed during early-phase clinical trials [14, 61]. In fact, up to now only two HDACis have been approved for the treatment of cutaneous T cell lymphoma: SAHA (Zolinza) in 2006 and Romidepsin/FK-228 in 2009. In 2011 the depsipeptide FK-228 has been further approved for the treatment of peripheral T-cell lymphoma [15]. Considering the recent evidences about a pro-oncogenic potential of class IIa HDACs [12, 37, 38, 62–64] and their impact on epigenetics [65], a stratagem to circumvent the side effects of class I HDACs inhibitors might consist in targeting class IIa HDACs.

Theoretically, targeting class IIa HDACs with specific inhibitors has three major drawbacks:

1. The high similarity of the catalytic site of these proteins to class I HDACs, which makes selective targeting rather difficult to achieve;
2. The formal question about the legitimacy of hitting the catalytic site of proteins that are almost enzymatically inactive against acetylated lysines. About this consideration the work of Bottomley et al. [31] explains how targeting of the catalytic site of class IIa HDACs and in particular the Zn^{2+} atom could impact on the structure of the C-terminus of the proteins, thus compromising their capability to interact with the super complex HDAC3/N-CoR/SMRT. Therefore, targeting class IIa HDAC domain could be an indirect strategy to impact on class I HDACs. By releasing

only class IIa driven deacetylation, a more selected transcriptional re-setting can be achieved, which could favor a drop in toxicity.

3. The methodological approach to measure class IIa HDAC inhibition. Up to now the best-characterized substrate for probing the elusive catalytic activity of vertebrate class IIa histone deacetylases is trifluoroacetyl-lysine [25, 66]. The activity of class I HDACs towards this molecule is indiscernible. Its use as a substrate for the validation of an inhibitor efficiency could exclude all class I HDACs as off-targets. Class IIa HDAC enzymatic activity measured with other methods or with classical substrates (e.g., acetylated H3) or commercial assays, generally based on acetyl-Lys, is extremely low when recombinant proteins are used [24]. Instead, when class IIa are purified from vertebrates the enzymatic activity can be provided by associated class I or IIb enzymes [24, 25, 31, 67]. Therefore, a double check approach should be used to test the potency and specificity of a class IIa HDACis. The potency of the compound should be evaluated by employing trifluoroacetyl-lysine, as a class IIa specific substrate, while its inhibitory activity against other HDAC classes should be excluded using "classical" substrates, such as acetylated lysines. A simplified screening could take advantage from the recently developed trifluoroacetyl-lysine derivative, a trifluoro acetyl-lysine tripeptide named substrate 6, which can be processed by all HDACs, with the exclusion of HDAC10 and 11. This molecule looks like a promising tool for single-run screening aimed to isolate/characterize subtype specific HDACis [68].

Class IIa inhibitors

Three different peculiarities of class IIa HDACs have been exploited to design specific inhibitors:

a. The catalytic site, and in particular the Zn^{2+} atoms.
b. The nuclear/cytoplasmic shuttling.
c. The N-terminal region and the binding to specific partners, such as the MEF2 family of TFs.

Targeting the Zn^{2+} binding domain

In accordance to the connecting unit (CU) linker chelator pharmacophore model [16, 69], a classical HDACi is composed of three parts [17]:

1. The MBG (metal binding group or zinc binding group ZBG), which is a group capable of chelating the Zn^{2+} in the catalytic site of HDACs (with the exception of sirtuins).
2. The connecting unit (CU), generally a linker hydrophobic region of five or more carbons, that mimics the acetyl-lysine. It could be linear or aromatic and it perfectly fits to the hydrophobic catalytic site of the targeted HDAC.
3. The CAP hydrophobic domain (usually aromatic) that interacts with aminoacids delimiting the border of the deacetylase catalytic site.

Slight modifications of the described structure impact both on the specificity and potency of the inhibitor.

The availability of the crystal structure of the class IIa deacetylase domain [29, 31] has encouraged the development and synthesis of many hydroxamates stemmed from SAHA, with the purpose of selectively influencing class IIa HDACs. In particular to improve specificity, many efforts have been spent in the modification of the CAP and of the ZBG of SAHA. In principle, the selective targeting of class IIa HDACs would require only some changes in the linker region, to better fit the peculiar catalytic site of class IIa HDACs. A recent study effectively demonstrated that slight modifications only in the linker region of SAHA increase the selectivity towards class IIa and class IIb HDACs [70]. However, the achieved results were not as promising as those obtained after modification of both the CAP and the linker region of SAHA [71]. This double tuning seems to be the better strategy to produce SAHA derivatives specific for class IIa HDACs. In a next future, new generation class IIa HDACis could stem from Tasquinimod (described below) that selectively targets the "structural" and not the "catalytic" Zn^{2+}. This peculiarity should increase the specificity because, as discussed above, this "structural zinc" is unique of class IIa HDACs. A summary of the literature data is shown in Fig. 3.

The most characterized of these hydroxamate-like drugs, are:

– MC1568 and MC1575 (Fig. 3, please note that in Fig. 3 we provide for MC1568 the recently reassigned structure [72]) are two class II HDACs inhibitors specific for HDAC4 and HDAC6 [73–76]. They are derivatives of classical class I HDACs inhibitors aroyl-pyrrolyl–hydroxyamides (APHAs), showing selectivity towards class IIa HDACs. The modified linker region provides this selectivity. Compared to the original class I inhibitors, they exhibit a decreased cytotoxic effect [73]. Despite this fact, MC1568 and MC1575 show some cytostatic effects in melanoma cells [76] and in ER + breast cancer cells [74]. The anti-proliferative effect is provoked by a block in the G1 phase of the cell cycle, through the induction of the Cdk inhibitor p21/Cip1/Waf1 [74]. MC1568 efficacy

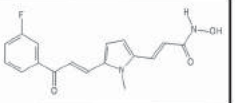
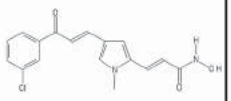
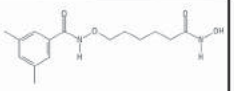
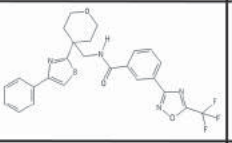
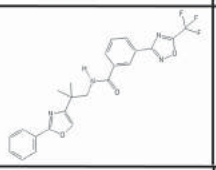
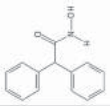
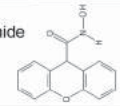
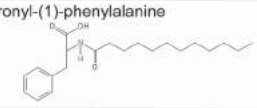
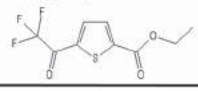
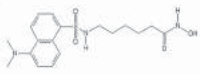
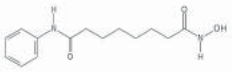
		IC50 (μM)												
		CLASS I				CLASS IIa				CLASS IIb		CLASS IV		
Name	Structure	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11	Substrate	Re.
MC1568		38.72	NA	NA	NA	0.22	0.22	0.22	0.22	0.22	NA	NA	Acetyl.pep	[73] [75]
MC1575		>20	>20	>20	>20	5	NA	NA	NA	NA	NA	NA	Class I Acetyl-H3 Class IIa Trifluoroacetyl Lys	[74]
LMK235		0.320	0.881	NA	1.278	0.0119	0.00422	NA	NA	0.0557	NA	0.852	Class I ac-H3 Class IIa Boc-Lys trifluoro-acetyl-AMC Class IIb and IV ac-p53	[71]
TMP269		>100	>100	>100	4.2	0.157	0.097	0.043	0.023	8,2	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9	[26]
TMP195		>100	>100	>100	11.7	0.111	0.106	0.046	0.009	47,8	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9	[26]
N-hydroxy-2,2 -diphenylacetamide		>10	6.06	NA	66	0.75	0.14	0.39	NA	>10	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(e-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide	[80]
N-hydroxy-9H -xanthene-9-carboxamide		NA	NA	NA	NA	0.25	0.11	0.05	NA	NA	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide	[80]
N-lauronyl-(1)-phenylalanine		>100	NA	NA	NA	NA	NA	21	NA	>100	NA	NA	Boc-Lys-(Ac)-AMC	[81]
Ethyl 5-(trifluoracetyl) thiopene-2-carboxylate		5.7	NA	3.5	NA	0.32	NA	NA	NA	0.55	NA	NA	Fluor de Lys HDAC1,3 Trfluoroacetamide -Lys HDAC4,6	[67]
Compound 2		0.95	1.38	1.12	3.98	0.33	0.40	2.56	NA	0.13	0.42	0.48	Fluor de Lys	[85]
BML-210		37.06	22.76	5.09	>300	NA	NA	>300	NA	>300	>300	NA	Competition binding	[118]
SAHA		0.22	0.56	1.79	2.74	>10	1.3	>10	>10	0.027	0.11	0.082	Class I, IIb, IV Fluor de Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC	[80] [85]

Fig. 3 Structures and summary of the available literature data on the IC50 for the proposed class IIa inhibitors

in cancer cells finds rationality in the capability of up-regulating the tumor suppressor Brahma, repressed by HDAC9 [77]. Curiously, MC1568 has been reported stabilizing the HDAC4-MEF2D complex in differentiated C2C12 myoblasts, thus impairing instead of favoring myogenesis [78].

– LMK235 (N-((6-(hydroxyamino)-6-oxoh exyl)oxy)-3,5-dimethylbenzamide) is a hybrid between two

classes of class I HDACis: the hydroxamic acids and the benzamides (Fig. 3) [71]. The specificity towards HDAC4 and HDAC5 is conveyed by the hydrophobic dimethyl substituted phenyl ring, which acts as a CAP group, matching class IIa active site better than class I [71]. This modification makes the molecule less toxic and more suitable for the treatment of some malignances, when compared to class I HDACis. Furthermore, LMK235 is able to re-sensitize cancer cells to cisplatin, better than SAHA [71].

- TMP269 and 195 (Figs. 3, 5) are two recently developed class IIa HDACis in which the classical hydroxamic Zn^{2+} binding domain is substituted by a trifluoromethyloxadiazolyl group (TFMO) [26] that highly resembles the trifluoromethylketone (TFMK) adopted by Bottomley and colleagues in their biochemical study of the ZBD [31]. The ring structure of the TFMO group increases its stability with respect to the highly unstable TFMK series of compounds [79]. Moreover, this TFMO moiety, differently from hydroxamate, acts as a non-chelating metal binding group, which interacts with the "catalytic" Zn^{2+}, through weak electrostatic interactions. As a consequence, the TFMO series has fewer off-targets compared to hydroxamates. Augmented selectivity is indirectly proved by gene expression profile studies in (PHA)-activated human peripheral blood mononuclear cells (PBMC) (Fig. 5). In these cells SAHA modulates the expression of 4,556 genes, whereas TMP195 regulates only 76 genes [26]. Curiously this finding is in accordance to what was observed in fibroblasts, where HDAC4 directly modulate only 76 genes [38]. To better characterize the transcriptome profile induced by their TFMO series of compounds, Lobera and colleagues purified T cells (CD3+), B cells (CD19+) and monocytes (CD14+) from the PHA-stimulated PBMC population and separately treated the three subpopulations with TMP195. T and B cells turned out to be very low sensitive to TMP195 (17 and 36 genes regulated, respectively); on the contrary the effect of the compound on monocytes was impressive (587 genes) and was not due to an increase in the expression of class IIa HDACs in these cells compared to the other two cell types. In particular the inhibitor interfered with monocytes to macrophages M-CSF (macrophage colony-stimulating factor)-induced differentiation. These findings candidate class IIa HDACs as druggable targets for immunological diseases [18, 71].
- *N*-hydroxy-2,2-diphenylacetamide and *N*-hydroxy-9H-xanthene-9-carboxamide (respectively, compound 6 and 13 in the original manuscript) are two diphenylmethylene hydroxamic acids characterized by Besterman group as class IIa HDACs specific inhibitors active in the μM range [80]. Both molecules exhibit a certain degree of symmetry and the second compound could be considered as the rigidification of the diphenyl moiety of the first (Fig. 3). This modification increases the specificity of the molecule towards HDAC7 [80].
- *N*-lauroyl-(l)-phenylalanine is a class IIa HDACi active in the μM range (Fig. 3) [81]. It was identified during a screening of a commercial available library of compounds. The specificity was scored not merely by classical measurements of HDAC activity but also through a fluorescence assay, which exploits the competition between a fluorescent substrate and the putative inhibitor for each purified HDAC [81]. This molecule shows anti-tumoral properties against ER+ breast cancer cells and can influence the expression of some MEF2-target genes (Fig. 5) [37].
- Ethyl 5-(trifluoroacetyl)thiophene-2-carboxylate [67] is the founder of a class of compounds, the trifluoroacetylthiophenes, that targets class II HDACs (class IIa and HDAC6) with some specificity. It was identified during a screening of a commercially available library of compounds using both the wt and the GOF mutant of HDAC4 as targets. It is a tripartite molecule characterized by: (i) a trifluoromethyl ketone group that chelates the active site zinc in a bidentate manner, (ii) the central thiophene ring that fits perfectly to class IIa active site and (iii) the amide group that interacts with the surrounding residues. The chemistry and the trifunctional nature of this compound justify its specificity.
- Tasquinimod (Fig. 4) is a promising drug for the treatment of advanced castration resistant prostate cancers [82, 83]. It acts by perturbing the tumor microenvironment. Differently from the aforementioned molecules it was not rationally designed or screened to target HDACs. Nevertheless, this carboxamide is able to enter the ZBD of HDAC4, keeping it in the inactive form and thus reorganizing the HDAC4 catalytic site. Tasquinimod-induced structural changes are causative of N-CoR/SMRT/HDAC3 displacement [43]. This finding is surprisingly considering the pronounced steric hindrance of the molecule, which is profoundly different from all SAHA derivatives. However, by virtue of its selective targeting of the "structural" Zn^{2+}", Tasquinimod molecular backbone could substitute SAHA as starting model for the development of specific inhibitors. From a molecular point of view the inactivation of HDAC4 prevents HIF-1α deacetylation, thus inducing its destabilization. Clinically, in hypoxic conditions the activation of HIF-1α transcriptional program stimulates the differentiation of tumor infiltrating myeloid derived suppressor cells into tumor-associated macrophage,

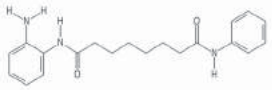
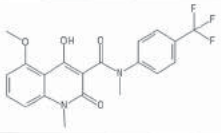
Name	Structure	DISPLACEMENT FROM PROTEIN BINDING IC50 (μM) HDAC4 - MEF2	HDAC4 - N-CoR	Assay	Re.
BML210		5	NA	Two-Hybrid in HeLa cells	[95]
Tasquinimod		NA	<1	Co-Ip in 293 cells	[43]

Fig. 4 Structure and binding interference properties of BML210 and Tasquinimod, two compounds capable of altering interaction of class IIa HDACs with their partners

which secrete pro-angiogenic factors [84]. Authors, therefore, proposed Tasquinimod as an anti-angiogenetic drug, which anti-cancer efficacy is being evaluated in pre-clinical models [43].

These last three molecules are considered unconventional inhibitors because, even though characterized by a tripartite motif, they are not SAHA derivatives.

Targeting the nuclear-cytoplasmic shuttling

In 2011, Brown group made the first attempt of blocking class II HDACs in the cytoplasm [85]. Starting from the structure of SAHA, they generated a couple of molecules by substituting the amino-phenyl group with a fluorescent dansyl group. This modification increases the specificity for class II HDACs in spite of a loss of reactivity against class I HDACs. If used in the μM range, the most effective molecule of the series, named compound 2 (Fig. 3), increases the fraction of cytoplasmic HDAC4 in prostate cancer cells PC3. The authors suggested that since the inhibitor accumulates in the cytoplasm, it binds HDAC4, thus impeding the interaction with importin-1α. As a consequence, the inhibitor increases the fraction of cells in the G1 phase of the cell cycle, the levels of p21/Cip1/Waf1, of acetylated H3 and tubulin. The increase of tubulin acetylation is probably due to the inhibition of HDAC6 [86] and seems to be unrelated to the suppression of class IIa [85].

It must be underlined that the IC50 values of these new inhibitors have been estimated by measuring the enzymatic activities of HDACs purified from mammalian cells, using the Fluor–de-Lys substrate [85]. Therefore, in the case of class IIa HDACs, it must be intended as indirect, deriving principally from the associated class I HDACs.

The strategy of interfering with class IIa HDACs nuclear accumulation could be attractive in oncology, as increasing evidences demonstrate that nuclear resident class IIa can display oncogenic functions [37, 38], but it might also present some drawbacks. First of all, class IIa HDACs possess also cytoplasmic functions [reviewed in 44], which could be amplified after inhibition of their nuclear import. Moreover, the cytoplasmic accumulation of class IIa HDACs is sometimes an indirect still uncertain effect of class I inhibition. For example the class I/II inhibitor LBH589, which is a SAHA derivate, confines HDAC4 in the cytoplasm in irradiated non-small cell lung cancer cells [87]. Considering all these drawbacks, the nucleus/cytoplasmic shuttling of class IIa HDACs seems to be the less druggable feature of these proteins.

Class IIa HDACs N-terminus, which allows their interaction with some partners, such as MEF2 family of TFs

As discussed above, class IIa HDACs' N-terminal region (Fig. 1) mediates the interaction with multiple partners and contains a glutamine-rich domain (with the exception of HDAC7) that allows homo- and heterodimerization among the different class IIa members [12, 88]. The best-characterized class IIa transcriptional partners are the MEF2s proteins [49, 89]. Several of the biological functions attributed to class IIa HDACs are the results of the MEF2s transcriptional repression [27, 37, 38]. The phenotype of the single knock-out of class IIa HDACs could be explained as the effect of MEF2 over-activation in bone (HDAC4), heart (HDAC5/9) and cardiovascular system (HDAC7), in relation to the district in which the single HDACs are more abundant [90–92]. Hence, the design of an inhibitor that displaces class IIa HDACs from MEF2s could be a good approach to selectively interfere with this specific repressive exploit. A limitation to this strategy concerns the promiscuity of the class IIa HDACs sequence required for this interaction (aa 166-184 in HDAC4). In fact, this stretch of amino acids is also involved in the interaction with additional partners, among which, the nucleoporin Nup155 [93] and the demethylase JARID1B [94] (Fig. 1). An alternative plan to influence the MEF2-

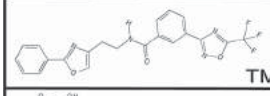
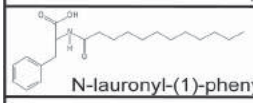
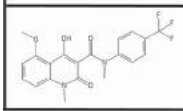
Compound	Cells	Treatment	Up-reg. MEF2-targets	Experiment	Re.
 TMP195	PBMCs PHA-stimulated	3μM 60 hrs	*ASB2,CCL1,ATP1B2*	DNA Microarray	[26]
 N-lauronyl-(1)-phenylalanine	MCF7	100μM 48 hrs	*KLF2, RHOB, NR4A1 KLF3, MARK1, GADD45γ*	qRT PCR	[37]
 BML-210	SK-UT-1 SK-LMA-1 DMR	10μM 36 hrs	*KLF2, RHOB, NR4A1*	qRT PCR	[38]
 Tasquinimod	LNCap	50μM 24 hrs	*IRS1, DTNA, ARRDC3 LHX4, KCNG1, ISL2*	DNA Microarray	[82]

Fig. 5 Summary of the available literature data on the effect of class IIa HDACs inhibitors on MEF2s-dependent transcription. MEF2s are the foremost characterized transcriptional partners of class IIa HDACs. Hence, an effect of these inhibitors on the expression of MEF2s target genes is an important read-out of their activity

HDAC axis could be targeting the region of MEF2s that interacts with class IIa HDACs. Using this approach, BML-210 (Figs. 3, 4, 5), a weak class I HDAC benzamide inhibitor, was found to interact through its aminophenyl-group with the hydrophobic residues of MEF2s (aa 66-69) thus displacing class IIa HDACs [95]. Using the crystal structure of the HDAC9–MEF2B complex as a guide [96], authors generated a panel of more powerful BML-210 derivatives. In the next future it will be important to further improve the specificity of these compounds to exclude residual targeting of class I HDACs.

Conclusions and perspectives

The identification of molecules that could reset the transcriptional profile in neoplastic cells has raised many hopes for new anti-cancer therapies [97]. Unfortunately today this goal has been only partially reached. Nevertheless an epigenetic therapy against cancer is still subject of intense research. A new impetus in this field was given by the discovery of the demethylases [98, 99] and the synthesis of their specific inhibitors [100]. A more niche-research concerns class IIa HDACs and their selective inhibitors, which are hypothesized to be less powerful than pan-HDACis but more specific. However, these studies are still in their infancy and the applicability of class IIa HDACis in clinic requires still intense laboratory characterization. Additional experiments and data are mandatory to characterize and understand the contribution of these molecules to epigenetic changes in vivo. Up to now, information about the impact of class IIa HDACis on RNA non-coding world and the role of class IIa HDACs in stemness maintenance are very limited [101]. In parallel the efforts trying to design, isolate and characterize new compounds, acting as epigenetic regulators must persist. In addition, a robust in vitro pre-clinical characterization of molecules already available is needed to define: their molecular mechanism of action, their ideal context of utilization and off-targets effects. All these efforts are justified by the benefits that drug-induced genetic reprogramming could exert on different diseases.

Certainly anti-cancer therapy is the first and most important scope. Nevertheless, the involvement of class IIa HDACs in the regulation of Glut4 [102–105], of the NF-kB pathway [106, 107] and of many neuronal activities [108–111] could stimulate studies about the employment of class IIa HDACis for the treatment of diseases other than cancer, such as diabetes [112], neurodegenerative disorders [113, 114] and inflammatory diseases [26, 115–118]. There are opportunities out there; we just have to find out what is the best compound for each specific application.

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

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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, 10^4 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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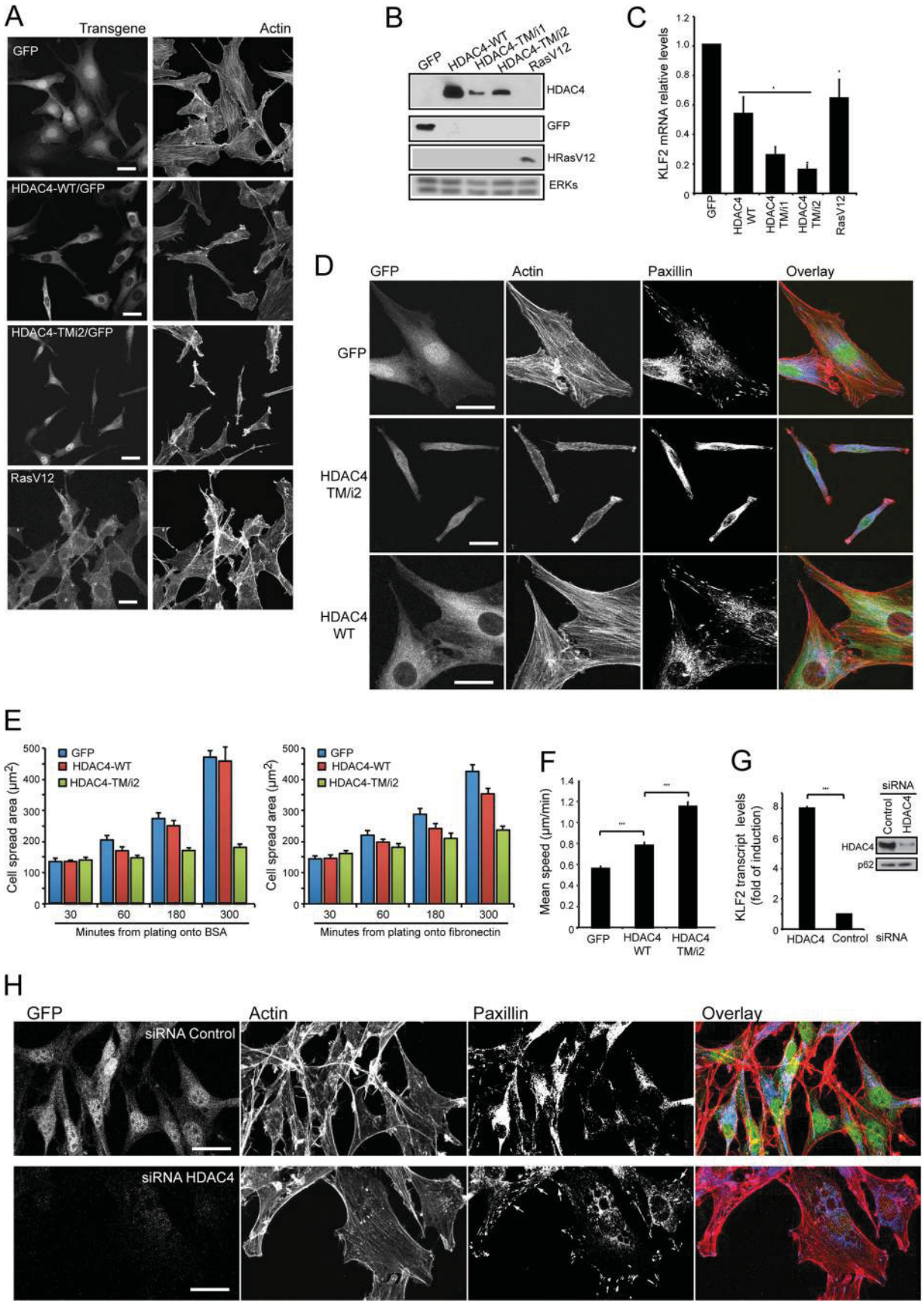
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Transgene
Actin
GFP
HDAC4-WT/GFP
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B
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Actin
Paxillin
Overlay
GFP
HDAC4 TM/i2
HDAC4 WT
E
GFP
HDAC4-WT
HDAC4-TM/i2
Cell spread area (μm²)
Minutes from plating onto BSA
Minutes from plating onto fibronectin
F
Mean speed (μm/min)
GFP
HDAC4 WT
HDAC4 TM/i2
G
KLF2 transcript levels (fold of induction)
siRNA
Control
HDAC4
HDAC4
p62
HDAC4
Control
siRNA
H
GFP
Actin
Paxillin
Overlay
siRNA Control
siRNA HDAC4

(BD Transduction Laboratories), and anti-Erk, ant-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 β_2-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 to detect 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. *, $P < 0.05$; ***, $P < 0.001$.

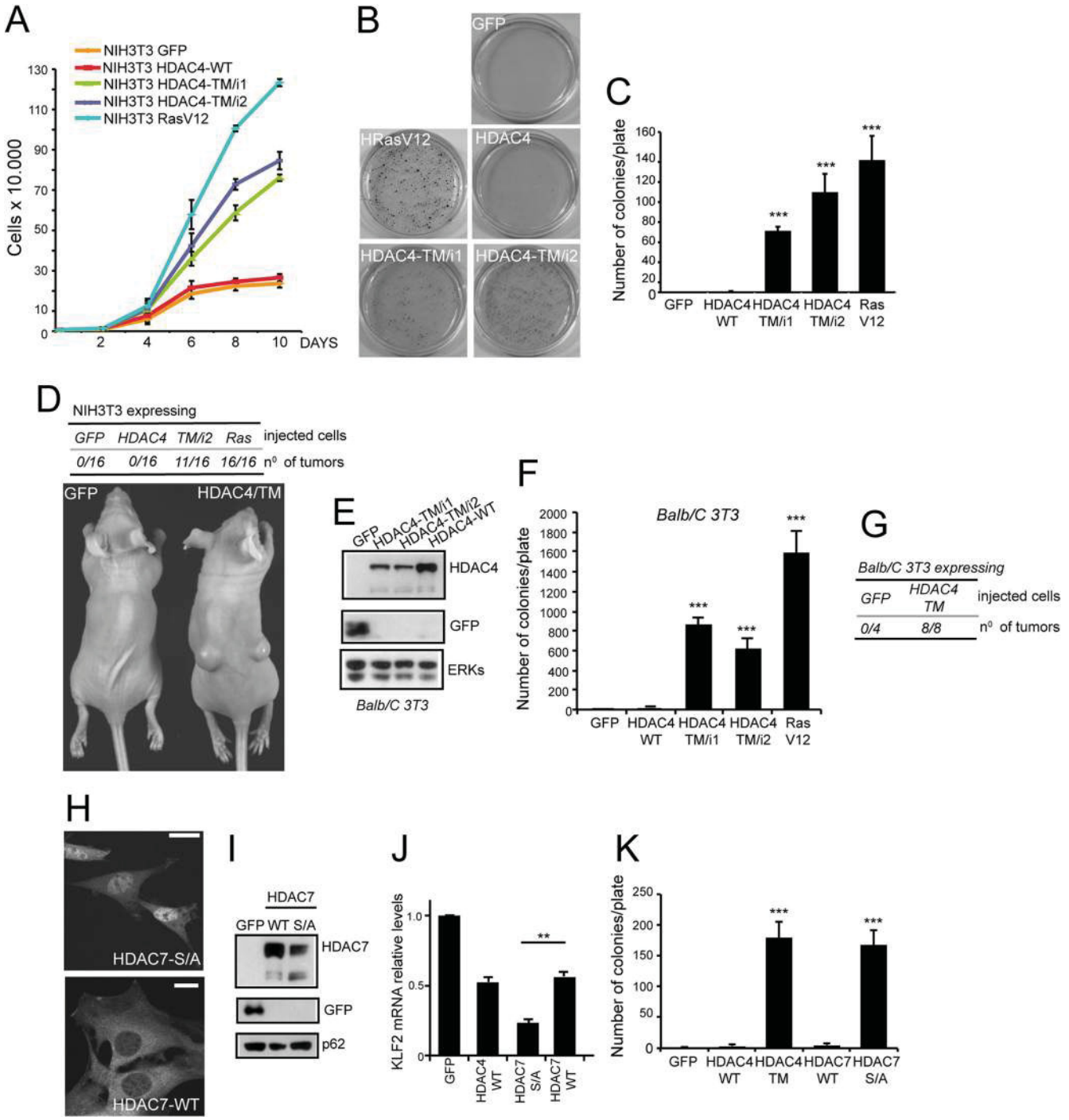


FIG 2 Transforming ability of HDAC4/TM. (A) NIH 3T3 cells expressing the indicated transgenes were grown in DMEM supplemented with 10% FBS. (B) Growth in soft agar of NIH 3T3 expressing the indicated transgenes, foci were stained with MTT. (C) Quantitative results of colony formation. (D) Analysis of 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 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 μm. (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. **, $P < 0.01$; ***, $P < 0.001$.

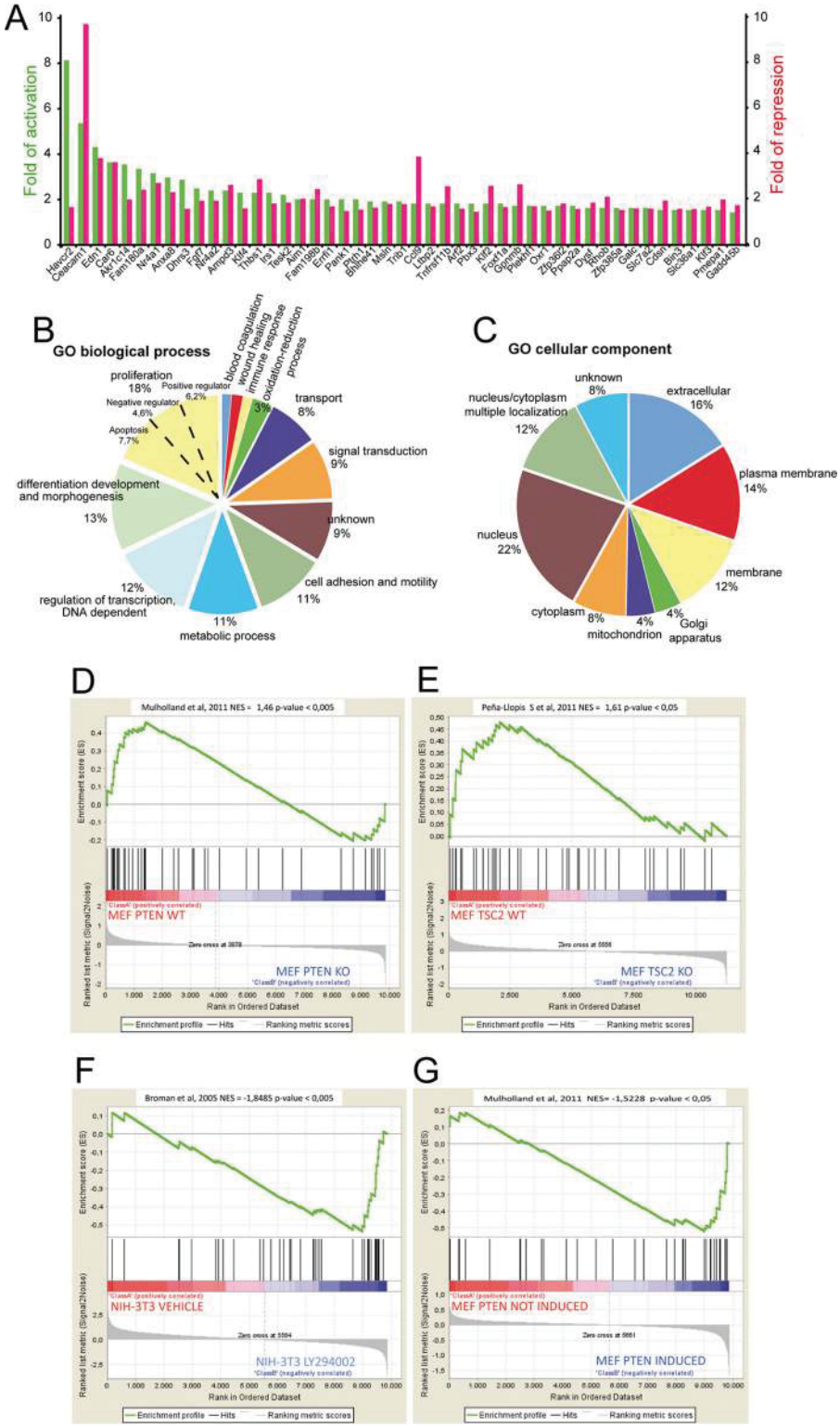
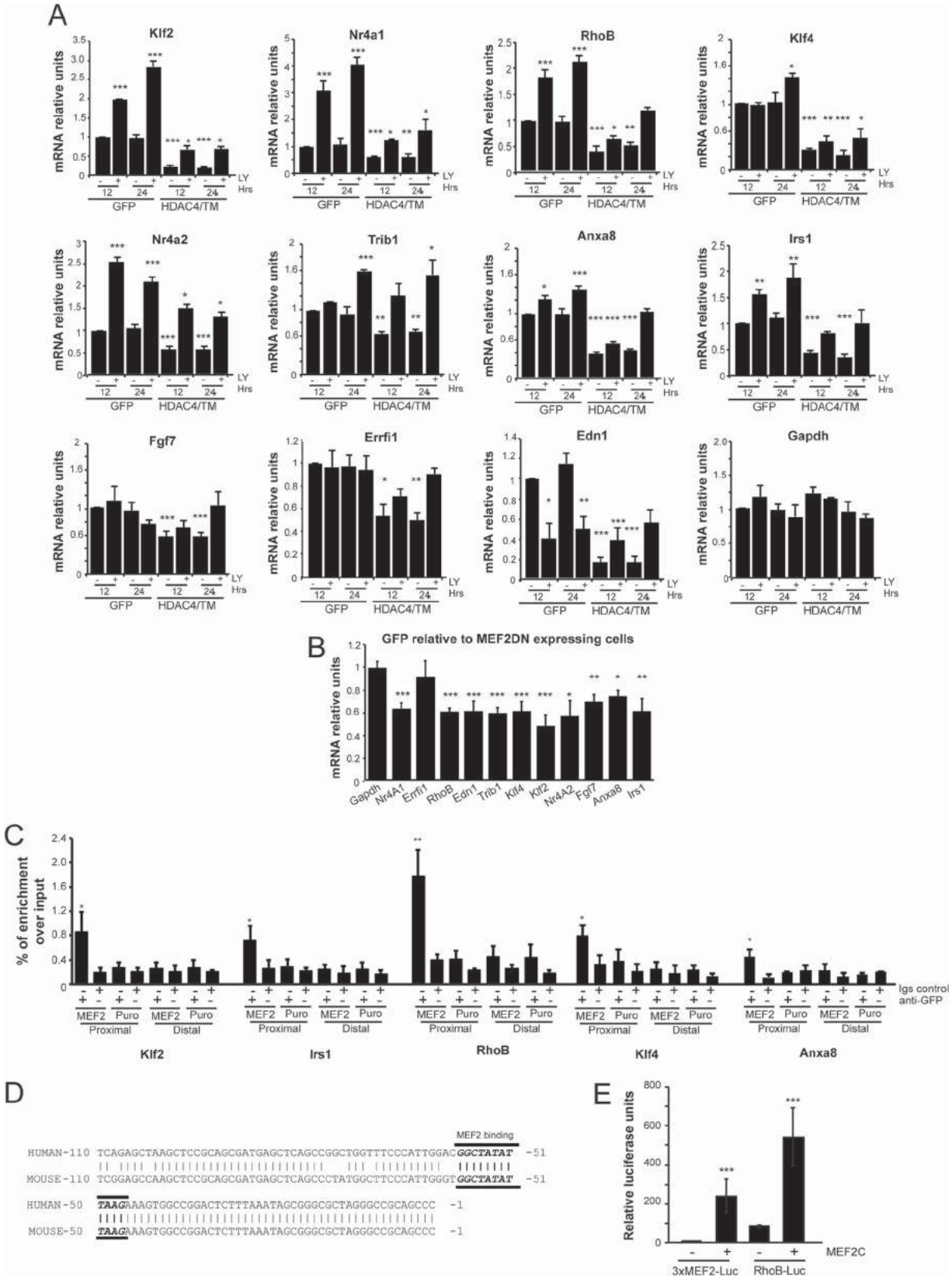


FIG 3 Identification of genes repressed by HDAC4/TM. (A) Diagram representation of the HDAC4/TM target genes. Microarray analyses were performed on GFP- and HDAC4/TM-expressing cells (repressed genes are indicated in red) and in HDAC4/TM cells transfected with control siRNA and the same cells transfected 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.


A
Klf2
Nr4a1
RhoB
Klf4
Nr4a2
Trib1
Anxa8
Irs1
Fgf7
Errfi1
Edn1
Gapdh
mRNA relative units
LY
Hrs
GFP
HDAC4/TM
B
GFP relative to MEF2DN expressing cells
mRNA relative units
Gapdh
Nr4A1
Errfi1
RhoB
Edn1
Trib1
Klf4
Klf2
Nr4A2
Fgf7
Anxa8
Irs1
C
% of enrichment over input
MEF2
Puro
Proximal
Distal
Igs control
anti-GFP
Klf2
Irs1
RhoB
Klf4
Anxa8
D
MEF2 binding
HUMAN-110 TCAGAGCTAAGCTCCGCAGCGATGAGCTCAGCCGGCTGGTTTCCCATTGGACGGCTATAT -51
MOUSE-110 TCGGAGCCAAGCTCCGCAGCGATGAGCTCAGCCCTATGGCTTCCCATTGGGTGGCTATAT -51
HUMAN-50 TAAGAAAGTGGCCGGACTCTTTAAATAGCGGGCGCTAGGGCCGCAGCCC -1
MOUSE-50 TAAGAAAGTGGCCGGACTCTTTAAATAGCGGGCGCTAGGGCCGCAGCCC -1
E
Relative luciferase units
MEF2C
3xMEF2-Luc
RhoB-Luc

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 $\log_2$ 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 subcellular 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 ± 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 μg) 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×MEF2-luc, together with *MEF2C*, as indicated. The *Renilla* luciferase plasmid was used as an internal control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

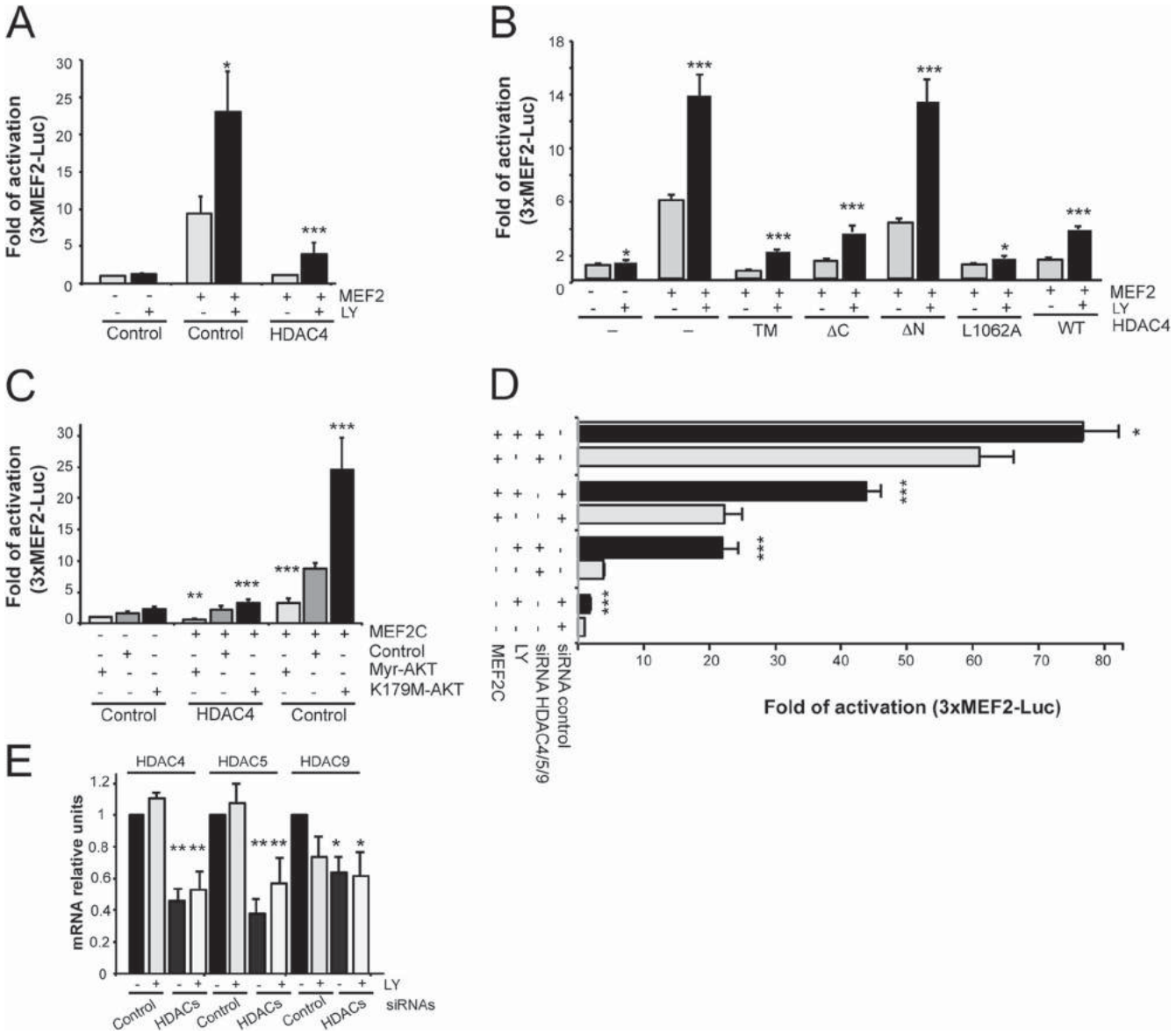


FIG 5 Regulation of the MEF2-dependent transcription by the PI3K/Akt pathway. (A) IMR90-E1A cells were transfected with the 3×MEF2-Luc (1 μg), the internal control pRL-CMV (20 ng), pcDNA3.1-HA-MEF2C (1 μg), and 300 ng of pEGFP expressing HDAC4. Cells were treated or not for 24 h with LY. (B) IMR90-E1A 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 μm/min (standard error of the mean [SEM] = 0.026; $n = 141$) compared to GFP-expressing cells

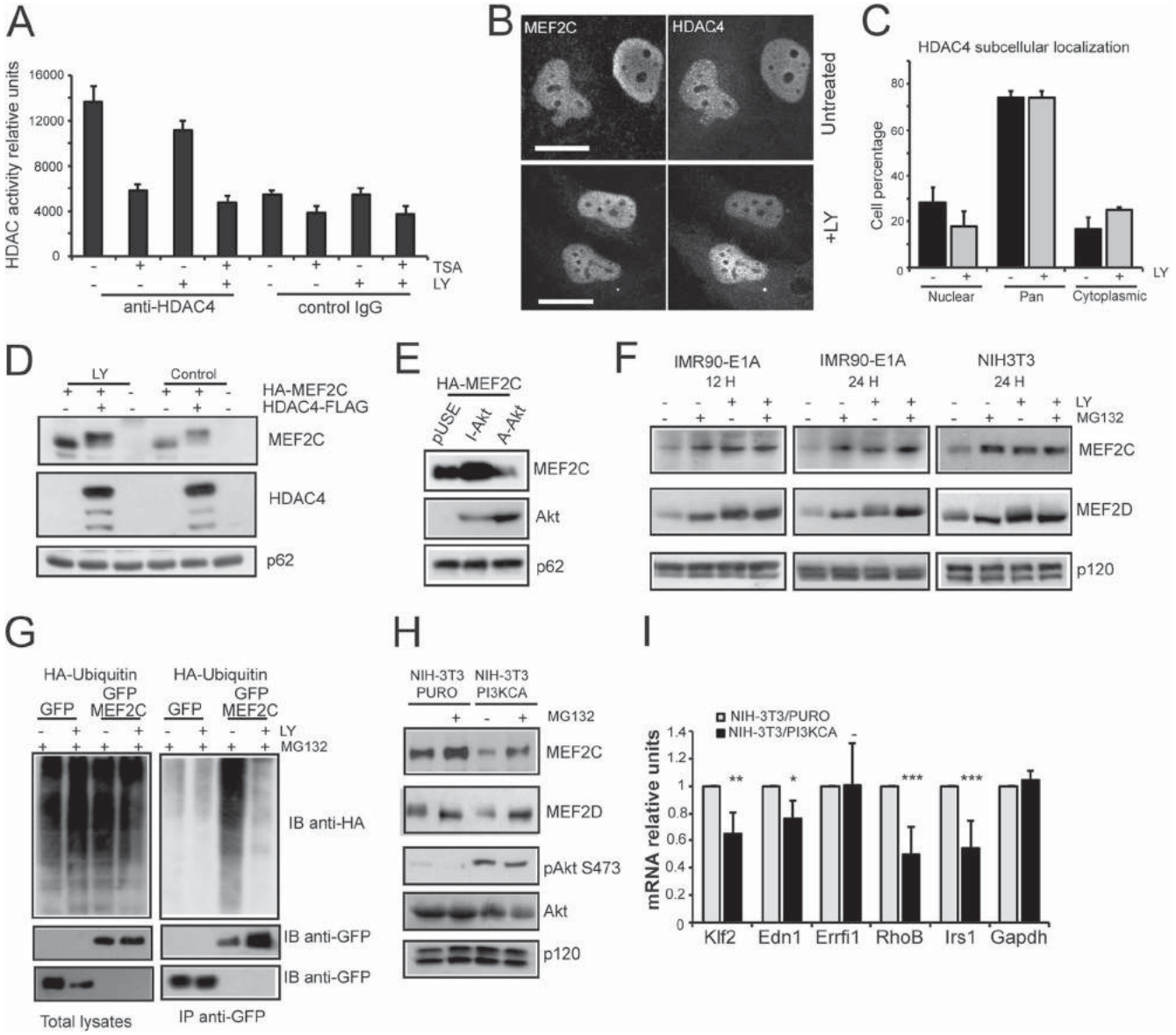
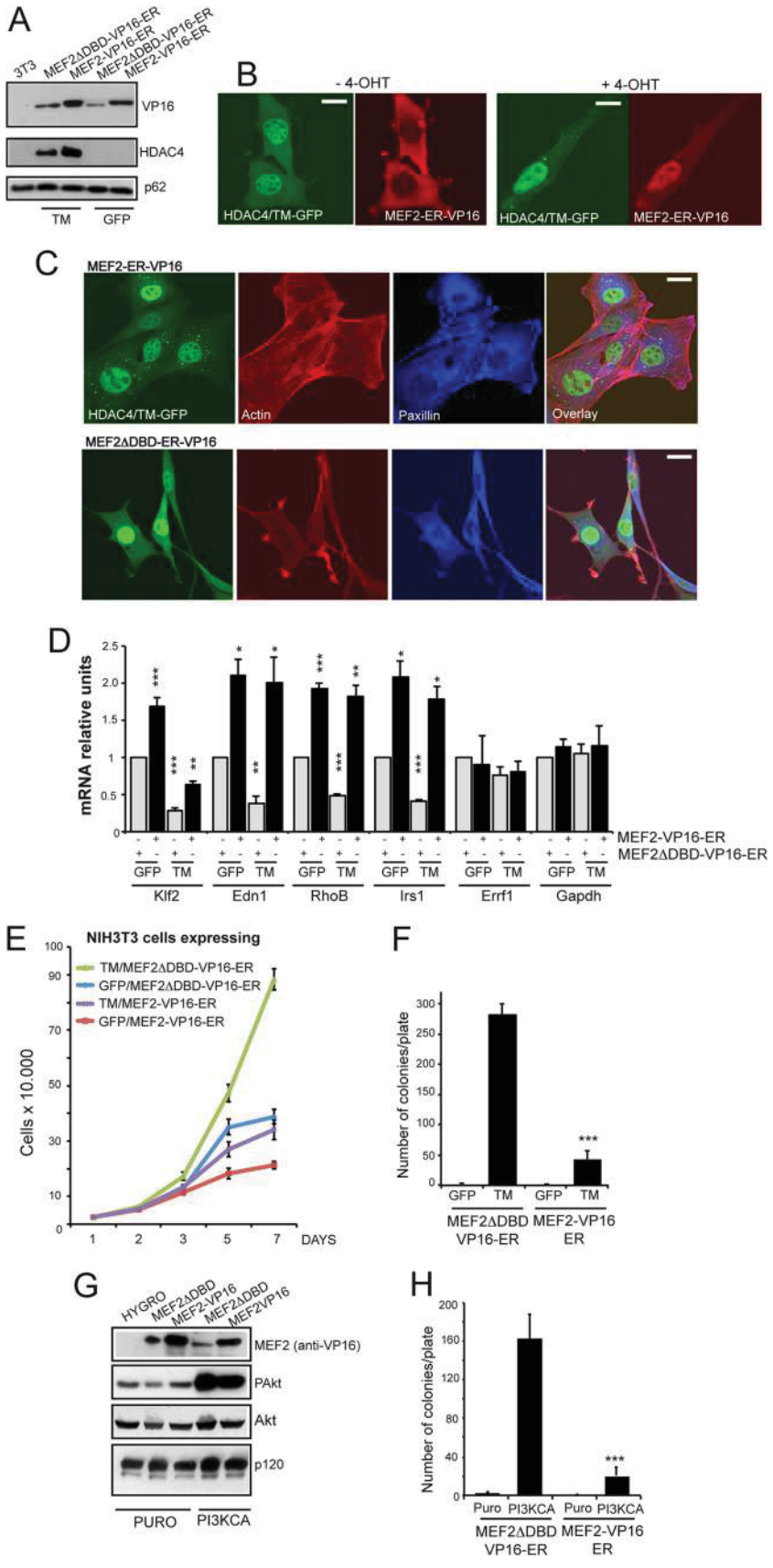


FIG 6 The PI3K/Akt pathway influences MEF2 protein stability. (A) HDAC4 was immunoprecipitated from NIH 3T3 cells treated or not for 18 h with LY. The HDAC activity was measured 15 min after the addition of the developer. (B) Confocal pictures of IMR90-E1A cells transfected with pcDNA3.1-HA-MEF2C (1 μg) 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 μm. (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 μg), 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 μg), and 1 μg 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 μm/min; SEM = 0.02; $n = 136$). This increase is significantly more pronounced in cells expressing HDAC4/TM (1.15 μm/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-


A
3T3
MEF2ΔDBD-VP16-ER
MEF2-VP16-ER
MEF2ΔDBD-VP16-ER
MEF2-VP16-ER
VP16
HDAC4
p62
TM
GFP
B
- 4-OHT
+ 4-OHT
HDAC4/TM-GFP
MEF2-ER-VP16
HDAC4/TM-GFP
MEF2-ER-VP16
C
MEF2-ER-VP16
HDAC4/TM-GFP
Actin
Paxillin
Overlay
MEF2ΔDBD-ER-VP16
D
mRNA relative units
MEF2-VP16-ER
MEF2ΔDBD-VP16-ER
GFP TM GFP TM GFP TM GFP TM GFP TM GFP TM
Klf2
Edn1
RhoB
Irs1
Errf1
Gapdh
E
NIH3T3 cells expressing
TM/MEF2ΔDBD-VP16-ER
GFP/MEF2ΔDBD-VP16-ER
TM/MEF2-VP16-ER
GFP/MEF2-VP16-ER
Cells x 10.000
DAYS
F
Number of colonies/plate
GFP TM GFP TM
MEF2ΔDBD VP16-ER
MEF2-VP16 ER
G
HYGRO
MEF2ΔDBD
MEF2-VP16
MEF2ΔDBD
MEF2VP16
MEF2 (anti-VP16)
PAkt
Akt
p120
PURO
PI3KCA
H
Number of colonies/plate
Puro PI3KCA Puro PI3KCA
MEF2ΔDBD VP16-ER
MEF2-VP16 ER

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 HDAC/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, microarray 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 *Errfi1*). *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*, *Errfi1*, 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 μm. (C) Confocal pictures of HDAC4/TM cells expressing MEF2-ER-VP16 chimera or its mutant defective in DNA binding MEF2ΔDBD-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 μm. (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 MEF2ΔDBD-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 MEF2ΔDBD-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-depedent 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.

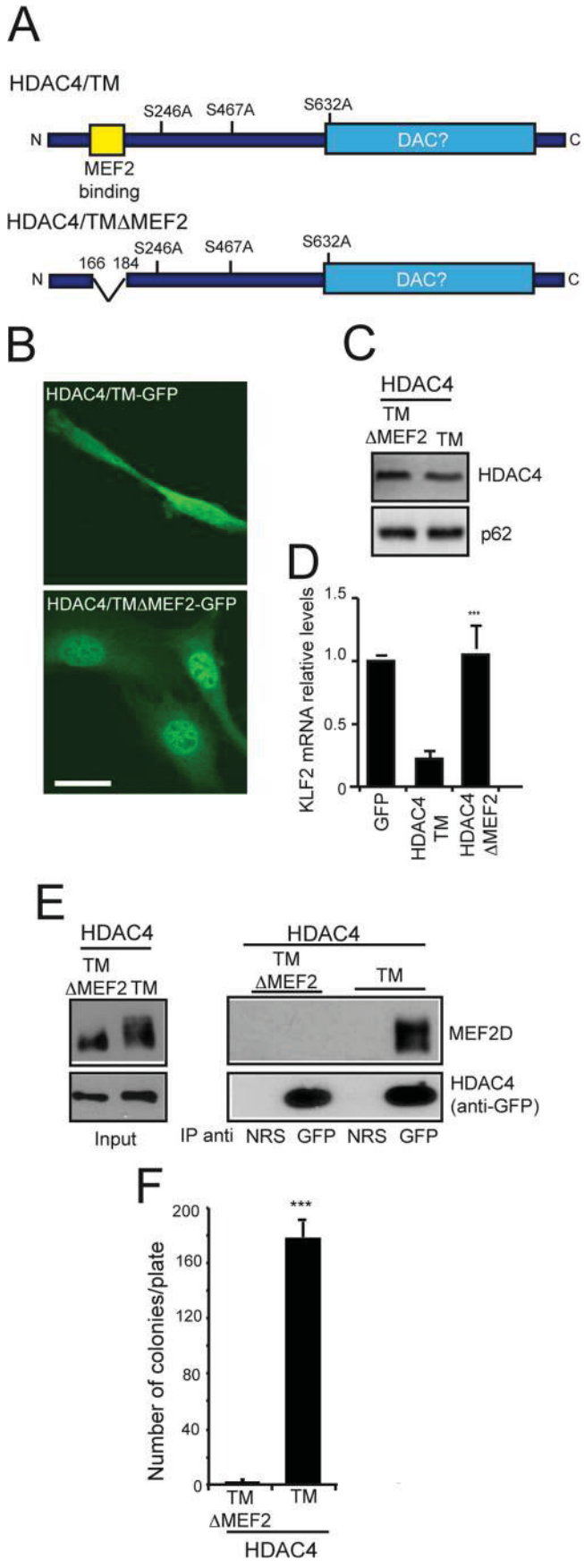
The PI3K/Akt pathway influences MEF2 protein stability. Although we have provided data that the PI3K/Akt pathway can influence MEF2 transcriptional activity, the mechanism involved remains obscure. In order to gain insight into the mechanisms exerted by PI3K/Akt signaling on MEF2s, we analyzed whether 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 addictive (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-transformed 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 (ΔDBD 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 MEF2ΔDBD-VP16 was ineffective (Fig. 7C).

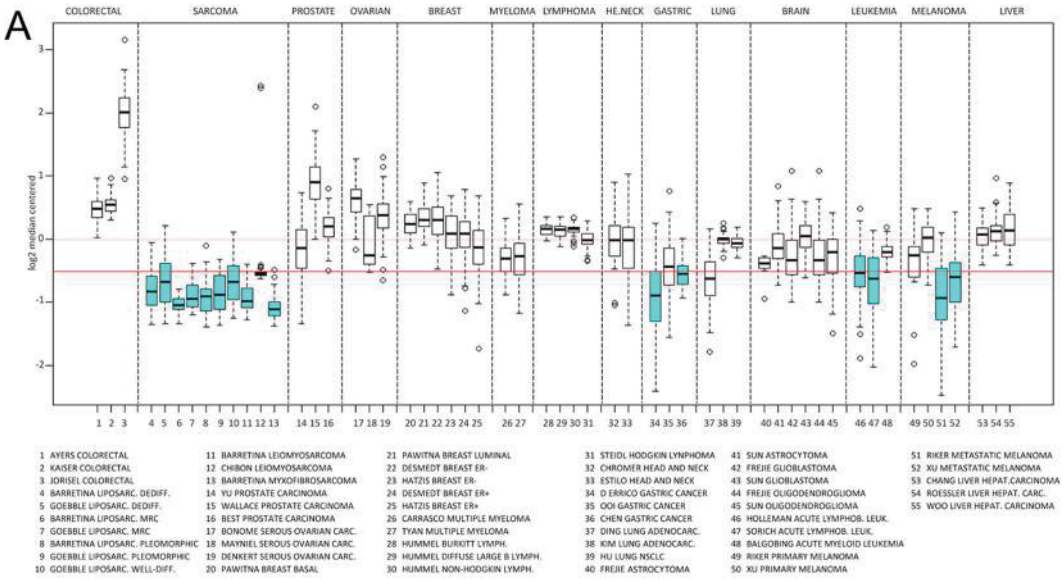
Induction of MEF2-VP16 but not of its deletion version (ΔDBD) elicited the upregulation of MEF2 target genes also in cells expressing HDAC4/TM (Fig. 7D). The reactivation of the MEF2 program was sufficient to dramatically limit the proliferative potential of cells expressing HDAC4/TM to a level similar 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-trasformed cells (Fig. 7G). Also in this case, the induction of MEF2-VP16 but not of its deleted version (ΔDBD) 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/TMΔMEF2 (Fig. 8A). NIH 3T3 cells expressing HDAC4/TM and HDAC4/TMΔMEF2 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/TMΔMEF2 mutant (Fig. 8E). Having characterized the properties of this new mutant, we next investigated its transforming ability. Figure 8F testifies that cells expressing HDAC4/TMΔMEF2 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 μm. (C) Immunoblot analysis of HDAC4/TM and HDAC4/TM/ΔMEF2 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/ΔMEF2 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. ***, $P < 0.001$.

A



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

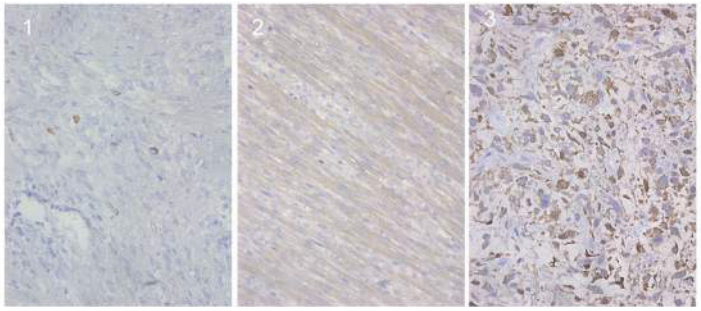
C **STS: Correlations of the expression levels (ρ) between the MEF2 signature and the indicated genes**

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

E



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 accumulation 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.

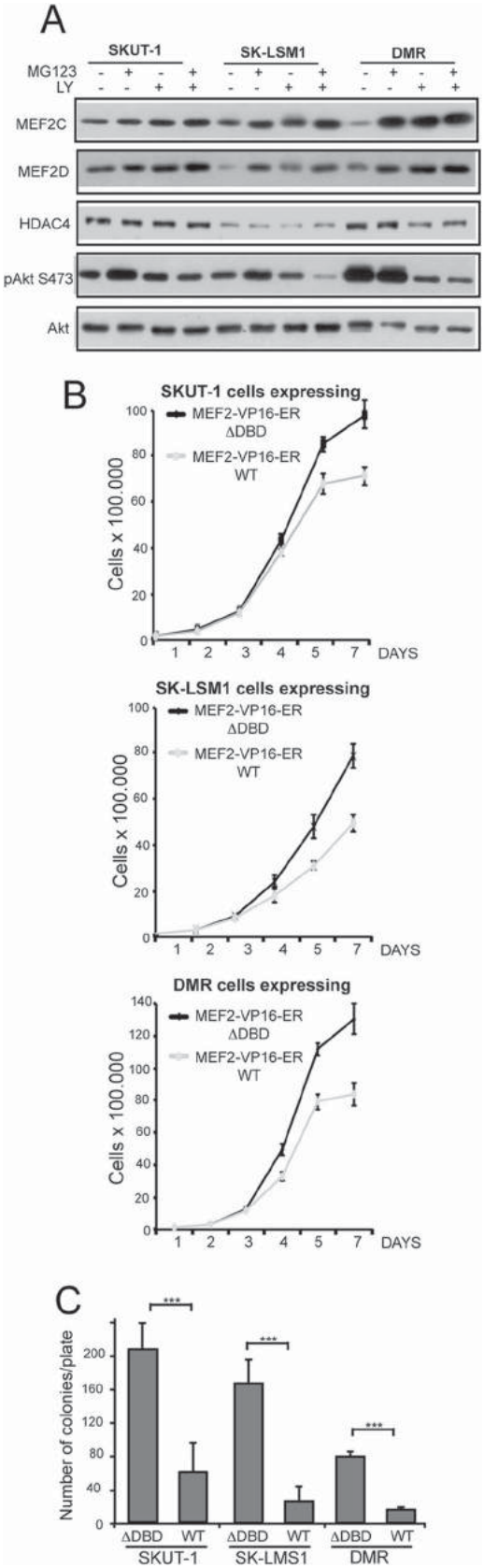
Pharmacological cotargeting of the PI3K/Akt pathway and of the MEF2-HDAC axis in sarcoma cells. To additionally prove the independent and synergistic action of HDAC4 and of the PI3K/Akt pathway on MEF2s and to evaluate the therapeutic perspective of our discovery, we used LY in conjunction with BML-210, 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(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 STS.

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. ***, $P < 0.001$.

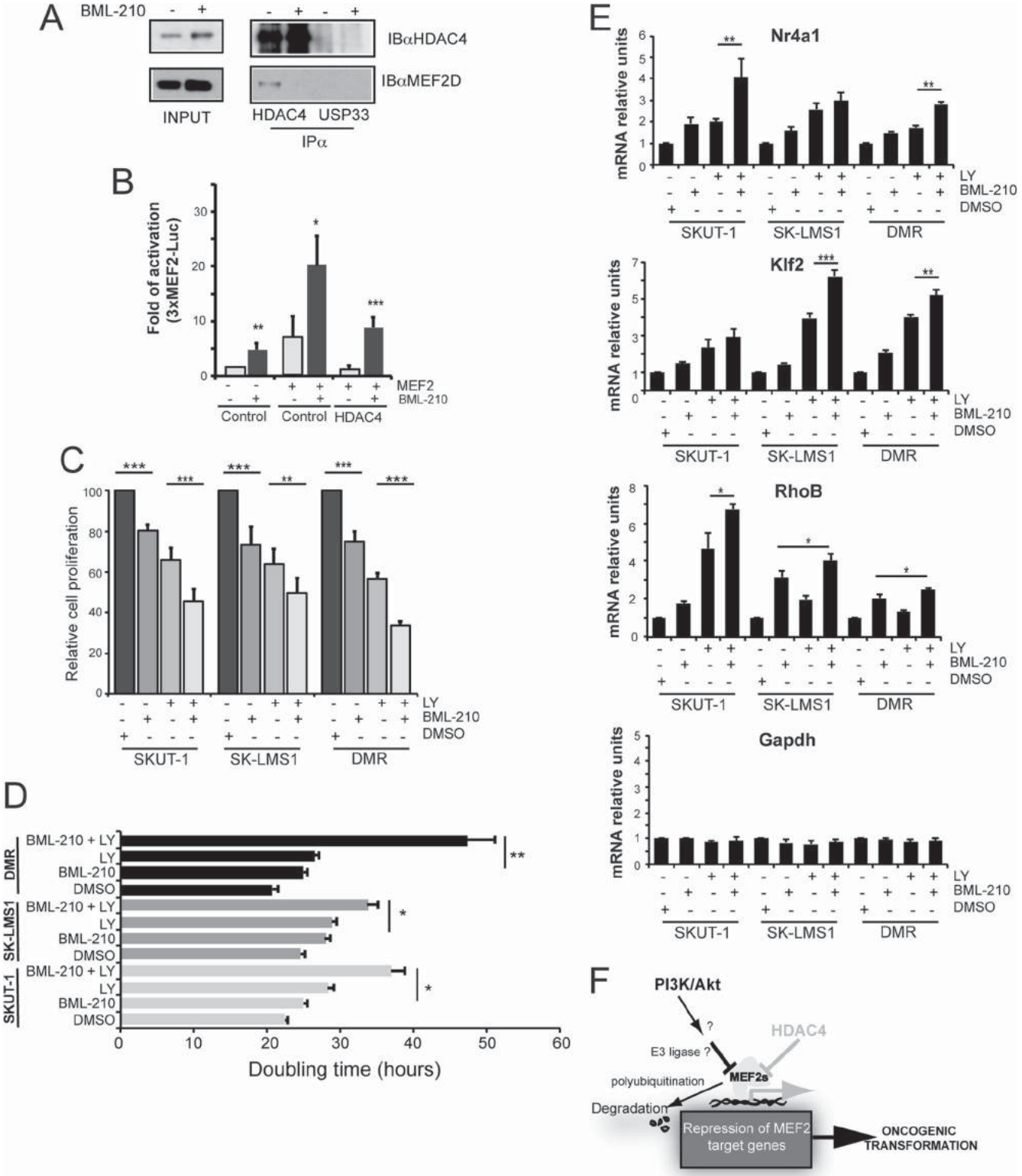


FIG 11 Pharmacological targeting of MEF2-HDAC axis and PI3K/Akt pathway. (A) Cellular lysates from IMR90-E1A cells treated or not for 36 h with BML-210 were immunoprecipitated with an anti-HDAC4 antibody. Immunoblots were performed with the anti-MEF2D and anti-HDAC4 antibodies. (B) IMR90-E1A cells were 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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Review

Beside the MEF2 axis: Unconventional functions of HDAC4

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ABSTRACT

The class IIa deacetylase HDAC4 is unequivocally known as a negative regulator of MEF2-dependent transcription. In the past years several works have allowed us to understand how different signals, mirroring specific environmental circumstances keep in check the repressive action of HDAC4 against MEF2s. At the same time, pieces of evidence have gradually accumulated about HDAC4 activities emancipated from MEF2s. The aim of this review is to discuss about these "unconventional functions" of HDAC4.

Contents

1. Introduction

Multicellular organisms coordinate their activities through an intricate network of signals that orchestrate different genetic programs for the benefit of the organism. These signaling networks operate during development to create and sculpture the different tissues. The same signaling networks are still operating in the adulthood, for the adaptation of the organism to the environmental incessant fluctuations.

Cells have evolved into different signal transduction pathways to link the nuclear response to specific extracellular events, with the final goal of targeting the transcriptional machinery. Intracellular signaling pathways, mainly through the fine-tuning of sequence specific DNA binding factors allow the reprogramming of the expressional landscape. Another level of control is operated on proteins defined as chromatin modifiers. These proteins influence the accessibility of transcription factors to DNA, by locally regulating chromatin architecture.

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Hence, cells have evolved into two integrated systems to re-program gene expression: the control of the transcriptional machinery and the control of the epigenetic changes.

Acetylation of lysines is an ancestral post-translational modification that can influence the activity of several proteins including histones, the molecular blocks on which DNA wraps around. Histone acetylation is a widespread epigenetic modification that marks active gene expression, by weakening the interaction of their positively charged tails with the negative backbone of DNA. This PTM is under the control of two competing enzymatic activities; the HAT (histone acetyl-transferase) and the HDAC (histone deacetylase) [1].

In humans the 18 HDACs are grouped into 5 different classes, based on sequence homologies to the yeast orthologs RPD3, HDA1 and SIR2 (classes I, IIa, IIb, III and IV). In this review we will discuss about HDAC4, which is a member of the class IIa subfamily. HDAC4, similarly to the other class IIa members (HDAC5, HDAC7 and HDAC9) is subjected to intense regulation by different signal transduction pathways in order to link changes in gene expression to the modified environmental conditions [2].

Class IIa HDAC cannot bind the DNA in a sequence specific manner but, they can be recruited on specific genomic regions following interactions with DNA-binding transcription factors. MEF2 (myocyte enhancer factor) transcription factors are the most characterized partners of class IIa HDACs [3]. A vast multitude of data reported in the literature has justified the view of these two distinct families of proteins, as a single transcriptional machinery, leading to the concept of the MEF2–HDAC axis. In addition to MEF2s, a long list of DNA binding factors, partners of class IIa HDACs have been identified and proposed. With this review we would like to discuss about some of these "unconventional partners" of class IIa members and of HDAC4 in particular.

2. Basic concepts on class IIa HDACs: things you need to know

Before addressing this discussion, we would like to summarize some basic concepts concerning class IIa HDACs:

1) Despite the fact that a certain level of expression of each class IIa HDACs can be detected in almost every tissue/cell, their levels can differ dramatically from tissue to tissue. Fluctuations in class IIa HDAC expression can be frequently observed in different cancer types [4] and among patients suffering of the same tumor [Clocchiatti et al., 2012 submitted].
2) In cultured cells class IIa HDACs can act promiscuously to repress gene transcription and compensatory mechanisms exist to overcome the dysregulation of one family member [5]. Redundancy and compensatory mechanisms have probably limited the impact of knocking down single members in mice.
3) Nevertheless genetic studies in mice have established that single class IIa HDACs can exert an irreplaceable role in specific tissues. This is the case of HDAC4 in the formation of hypertrophic cartilage and of HDAC7 in vascular endothelial cells [6,7].
4) Class IIa HDACs are subjected to multiple levels of regulation, including: transcriptional/translational control, ubiquitination/sumoylation, selective proteolysis, and phosphorylation at multiple sites. Cycles of phosphorylation/dephosphorylation control their nuclear/cytoplasmic shuttling and hence the repression/activation options [3,8].
5) Class IIa HDACs in vertebrates exhibit a very low deacetylase activity versus acetyl-lysines, whereas they can process tri-fluoroacetyl-lysine. This is the consequence of the substitution of the catalytic Tyr with His. Nevertheless, because of their ability to interact with other transcriptional co-repressors and "in primis" with the HDAC3/NCoR/SMRT complex, a lysine–deacetylase activity can be co-purified with class IIa HDACs from mammalian cells [9,10].

3. The MEF2–HDAC4 axis

The foremost-characterized partners of HDAC4 are the MEF2s, a MADS-box family of DNA binding transcription factors. The MAD and MEF domains lie in the amino-terminal region of the proteins and mediate binding to DNA, dimerization and cofactors recruitment. These domains are highly conserved among the different family members. By contrast, the carboxy-terminal half is much less conserved and contains the transactivation domain [11].

MEF2s play a myriad of roles during development as well as in the adult life. They control transcriptional circuits in cooperation with other transcription factors thus modulating the expression of a wide range of genes that varies from cell type to cell type. This genetic heterogeneity depends on the specific transcriptional frameworks, acting together with MEF2s on the different promoters/enhancers. Genes under the influence of MEF2s control differentiation, proliferation, morphogenesis, survival and apoptosis [12]. Not surprisingly MEF2-binding sites can be recognized in the promoters of more than 1000 genes. Since it is conceivable that undiscovered enhancers could be regulated by MEF2s, the number of genes under their control is likely to expand [13].

MEF2s are subjected to a diverse type of controls, which modulate their assembling into transcriptional active or silent complexes. The MAP kinase ERK5 binds to MEF2s and acts as a positive regulator [14]. On the opposite, class IIa HDACs can assemble, in a signal regulated manner, to repress MEF2 transactivation potential [15–17]. Comparative in vitro binding studies have testified that the affinity of interaction between HDAC4 and MEF2C or HDAC4 and other putative partners such as SRF and RUNX2 differ enormously, thus proposing that MEF2 is the preferred class IIa transcriptional partner [18]. The interaction with MEF2 involves the amino-terminal region of HDAC4 and more precisely a region encompassing aa168 and 185, conserved between class IIa HDAC members. This region interacts with amino acids 39–72 of MEF2 factors, spanning the junction of the MADS and MEF2 domains [15]. In a therapeutic perspective, the region of interaction was recently targeted by small molecules, to release class IIa-mediated repression [19].

Genetic studies have defined the essential role played by the MEF2–HDAC4 axis during the formation of the hypertrophic cartilage. Chondrocyte-specific deletion of MEF2c results in a lack of bone due to the failure in chondrocyte hypertrophy [20]. Defects in bone formation can also be observed in HDAC4-null mice, which die during the perinatal period due to severe growth retardations and numerous skeletal abnormalities, consequences of an excessive hypertrophic chondrocyte differentiation and inadequate endochondral ossification [6]. Alterations in the fine-tuning of the MEF2–HDAC4 axis could cause diseases in humans, as recently proposed to explain the neurodegeneration observed in the ataxia telangiectasia patients [21], in the brachydactyly mental retardation syndrome [22] or in specific types of cancer [Clocchiatti et al., 2012 submitted].

As introduced above, HDAC4 and class IIa can influence MEF2 transcription through different strategies:

i) Recruitment of other co-repressors including class I HDACs and in particular of the N-CoR/SMRT/HDAC3 complex [10,23]
ii) Competition for the binding to transcriptional activators
iii) Induction of PTMs such as phosphorylation-dependent sumoylation [24] that is repressive "per se" by hitting the same lysine residues that are regulated by acetylation [25].

Cells control the repressive influence of HDAC4 on MEF2-dependent transcription using different strategies. The best characterized is the phosphorylation-dependent control of its nuclear accumulation (Fig. 1) [4]. In addition alternative strategies linked to specific stimuli have evolved, as exemplified by the caspase-dependent cleavage operating during apoptosis or the PKA-regulated processing elicited by acute adrenergic stimulation in cardiomyocytes (Fig. 1) [26,27]. For an in-depth

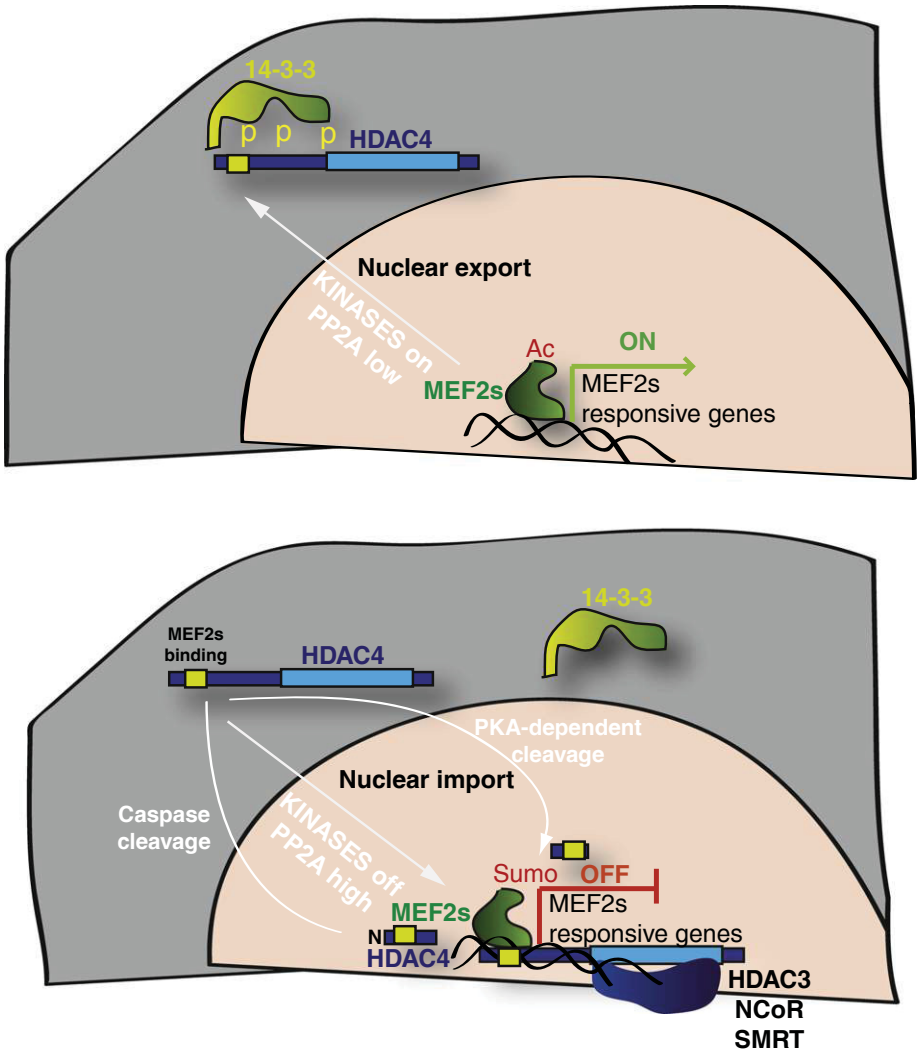

Fig. 1. Regulation of the MEF2–HDAC4 axis. Extracellular stimuli, acting through different serine–threonine kinases trigger the phosphorylation of HDAC4 and its association with 14-3-3 proteins, which favors cytosolic accumulation. On the opposite the serine–threonine phosphatase PP2A stimulates HDAC4 nuclear accumulation and the consequent repression of MEF2-dependent gene expression. Caspase-3-mediated cleavage during apoptosis or proteolytic processing supervised by PKA can elicit nuclear accumulation of HDAC4 amino-terminal fragments capable of repressing MEF2. Acetylation of MEF2 promotes transcription and is antagonized by HDAC4-dependent sumoylation. The HDAC3/NCoR complex is also shown. The MEF2 binding site and the deacetylase domain of HDAC4 are highlighted.

discussion about MEF2 and class IIa HDACs we refer the readers to other excellent published reviews [8,12].

4. HDAC4 and the hypoxia-inducible factor (HIF)

The fundamental controller of oxygen homeostasis HIF (hypoxia inducible factor) is a DNA binding transcription factor composed by the subunits α and β. The two subunits heterodimerize through the bHLH-PAS domains that are also involved in DNA binding. In vertebrates, HIF-2α is a paralog of HIF-1α, expressed in a cell-specific manner. Under normoxic conditions HIF-1α levels are continuously subjected to proteosomal degradation and hypoxia selectively suppresses this degradation. This key switch is orchestrated by the interplay between the oxygen sensor, the prolyl and asparaginyl hydroxylation (PHD) and the activity of the von Hippel–Lindau (VHL) protein, the substrate recognition element of an E3 ubiquitin ligase [28].

The first clues about an interaction between class IIa and HIF arose after a two-hybrid screening in yeast. The deacetylase domain of HDAC7 was isolated as an interactor of the ID domain of HIF-1α [29]. These initial studies in yeast suggested that HIF-1α shows preferences for some class IIa members. HIF-1α displayed a stronger binding for HDAC7 compared to HDAC4 and it was incapable of interacting with HDAC5. The carboxy-terminal region of HDAC7, including part of the catalytic domain was required for HIF-1α binding [29]. In a subsequent work, an interaction with HDAC5 was documented as well [30]. Since evidences that HIF-1α can be acetylated and that acetylation can regulate its stability were previously published [31] an interaction between HIF and HDACs was somehow expected.

A cautionary note for these studies is justified from the exclusive use of the overexpression that might give rise to false positives. Nevertheless it was observed that under hypoxic conditions, HDAC7 but not HDAC4 co-translocated in the nucleus with HIF-1α [29]. More surprisingly, HDAC7 increased the transcriptional activity of HIF-1 without affecting protein stability. To explain this paradox the authors suggested that HDAC7 is part of a complex recruiting also p300 [29]. Alternative mechanisms to explain the positive effect have also been proposed by another study. Binding of HIF-1α to class IIa HDACs can also favor the dissociation of FIH-1, a factor inhibiting HIF-1α [30].

At the level of the endogenous proteins, Qian and co-authors confirmed the binding between HIF-1α and HDAC4 [32]. The same authors also observed the interaction with HDAC6, a class IIb member, but not with HDAC7. siRNA-mediated dropping of HDAC4 and HDAC6 levels reduced HIF-1α protein expression and transcriptional

activity [32]. Overall this study indicates that HDAC4 and HDAC6 can influence HIF-1α stability in a VHL-independent manner [32]. As discussed above the discrepancy with the overexpression studies can be explained by different hypothesis: including the artifactual conditions, intrinsic to this approach but also the presence of HDAC7 specific PTMs or mutations in the used cell lines. A cell lineage effect could also explain the observations made in neurons. Here the HDAC4–HIF-1α axis but not HDAC6 seems to be important in the regulation of retinal neuron survival [33].

Interestingly, MEF2C is a transcriptional target of HIF-1α [34] and this regulation can play a role during cardiogenesis [35,36]. In a speculative view, class IIa HDACs in this differentiation pathway, could initially act as positive regulators of cardiogenesis, by supporting HIF actions, subsequently, they could buffer the response, by repressing MEF2C activity.

A further contribution to the role of HDAC4 in the regulation of HIF was recently discovered. Stable down-regulation of HDAC4 by shRNA augments acetylation of HIF-1α, decreases its stability and compromises the transcription of its target genes (VEGFα, lactate dehydrogenase A and glut1), whereas it does not influence HIF-2α [37]. Although this effect seems to be independent from HDAC1 and HDAC3, two class I enzymes able to assemble into a multiprotein complex together with HDAC4, it cannot be ignored that both class I enzymes should be down-regulated to exclude their involvement.

The regulation of HIF acetylation is complex and multifaceted with different lysines enrolled. These different acetylation events can be subjected to cell/tissue specific regulations [38]. Five lysine residues, within the N-terminal region have been proposed as targets of class IIa deacetylases [39]. Class III HDACs (sirtuins) can also modulate HIF acetylation. For example SIRT1 can deacetylate HIF-1α at Lys674, which is acetylated by PCAF. The final outcome of this activity is the inactivation of HIF-1α through the block of p300 recruitment [40].

HIF-1α can also be acetylated at Lys532. In this case acetylation, similarly to the N-terminal acetylation destabilizes the protein. Acetylation of Lys532 can be reverted by the recruitment of HDAC1 to HIF1α via MTA (metastasis-associated protein 1) [41]. On the other side, which is the acetyl-transferase handling Lys532 it is still a matter of debate [41]. Recently another acetyl-lysine at position 709 has been identified [39]. K709 acetylation is regulated by p300 and antagonized by HDAC1. It limits HIF-1α deacetylation, thus enhancing protein stability and transcriptional activity [37].

The importance of acetylation in the modulation of HIF activity is further emphasized by the discovery that E7 proteins, either from low and high-risk HPV (human papilloma virus) subtypes can stimulate HIF-1α dependent transcription. The activation of a pro-angiogenic response by HPV is important for a successful induction of warts, and in some cases, of cervical cancer. E7 binds the carboxy-terminus of HIF-1α and promotes the displacement of HDACs, including HDAC4 and HDAC7 from HIF-1α [42]. This supplanting activity correlates with the potentiation of HIF-1α functions.

In conclusions different groups have reported about physical interactions between class IIa HDACs and HIF-1. Evidences are stronger in the case of HDAC4. In terms of contribution to HIF-1α acetylation, stability and function, data are sometimes in contradiction and a common picture cannot be depicted. Hence, the precise mechanism under HDAC4 supervision, in the context of HIF activities deserves further studies. A question, which might not be simple to answer, since lysines of HIF-1α subjected to acetylation could change, from cell lineage to cell lineage and in relation to the different environmental conditions.

5. HDAC4 and STAT1

STAT (signal transducer and activator of transcription) are latent cytoplasmic transcription factors, which are activated by tyrosine phosphorylation in response to different stimuli including extracellular signals, such as cytokines and growth factors, or oncogenic lesions [43]. Despite tyrosine phosphorylation is a key switch in the control of these transcription factors, several hints point to a regulation of STAT activities through acetylation, independently from phosphorylation [44]. For example STAT3 acetylation influences inflammatory and proliferative responses [45,46] and class I HDACs play an important role in the control of STAT3 acetylation levels.

Recent discoveries highlight also the contributions of class IIa HDACs to the supervision of STAT1 activation. HDAC4 was reported to activate or repress STAT1 activities through two distinct mechanisms. Activation is mediated by direct deacetylation of aa321 nn, whereas repression engages sumoylation, through the involvement of another repressor [45,46]. The proposed models are still debated and further experimental work is necessary to clarify the conflicting aspects. STAT1 modulation by HDAC4 can be taken as an example of the capability of HDAC4 to act as a true deacetylase, as a SUMO-E3 ligase or being part of complexes carrying these enzymatic activities (Fig. 2).

In ovarian cancer cells resistant to cisplatin HDAC4 emerges as an activator of STAT1 [47]. In particular, resistant cells overexpress both HDAC4 and STAT1 and their depletion is sufficient to re-sensitize cells to the drug. By contrast no effect is reported on sensitive cells. Since the double silencing of HDAC4 and STAT1 has no additive effect in terms of cell death in resistant cells, Stronach and colleagues concluded that HDAC4 and STAT1 act in the same pathway. In particular, HDAC4 interacts with STAT1 only in resistant cells. Under these conditions HDAC4 deacetylates STAT1 thus promoting its phosphorylation and nuclear import, which results in a protective response, thanks to the stimulation of IRF1. HDAC4 down-regulation causes STAT1 acetylation and decreases its phosphorylation. The described regulation of STAT1 is in agreement with previous data obtained in 293 cells treated with IFNα [48]. However in this case the deacetylating enzyme was HDAC3 and not HDAC4 (with the important difference that HDAC4 acts independently from IFN pathway) [48]. Interestingly, deacetylation of STAT1 seems to occur in the cytoplasm and seems to be completely independent from HDAC3, as above discussed in the case of HIF-1α deacetylation.

The repressive influence of HDAC4 on STAT1 requires the involvement of the ligand activated nuclear receptors LXRα/NR1H3 and LXRβ/NR1H2, which play important roles in the control of cholesterol homeostasis and inflammatory responses [49].

In astrocytes the treatment with LXR agonists induces the binding of HDAC4 and PIAS1 respectively to LXRβ and LXRα and their consequent sumoylation [50]. Sumoylated LXRα/β binds nuclear pSTAT1 and forms a trimeric complex that contains HDAC4 and which prevents the binding of STAT1 to IRF1 promoter. In this manner LXR agonists block the IFN-y inflammatory response [50].

The role of HDAC4 as SUMO E3 ligase of LXRs and as a negative regulator of the inflammatory response was initially discovered in macrophages [51]. Here HDAC4 mediates sumoylation of both nuclear receptors LXRα and β, which allows the interaction with NCoR, thus preventing their ubiquitination and degradation. Therefore NCoR keeps iNOS (NO synthase) in a repressed status even in the presence of LPS [51] and this limits the killing potential of macrophages [52].

HDAC4 acts as the SUMO-E3 ligase that joins LXRα and β to SUMO-2/-3 and the down-regulation of HDAC4 is sufficient to abolish the sumoylation of LXR and the repression of iNOS, without the involvement of STAT1, that is instead necessary to repress iNOS in intestinal epithelial cells [53]. Furthermore, an indirect connection between class IIa HDACs and LXR was observed in adipocytes. Here the down-regulation of the LXR target GLUT4, in response to the signal that increases AMPc levels (such as fasting) depends on the occupancy by HDAC4 and HDAC5 of the LXR site on the GLUT4 promoter, rather than on sumoylation [54].

Further work is necessary to confirm these observations. Details on the mutual binding regions, identification of specific mutants and elucidation of the stoichiometry of the multiprotein complexes,

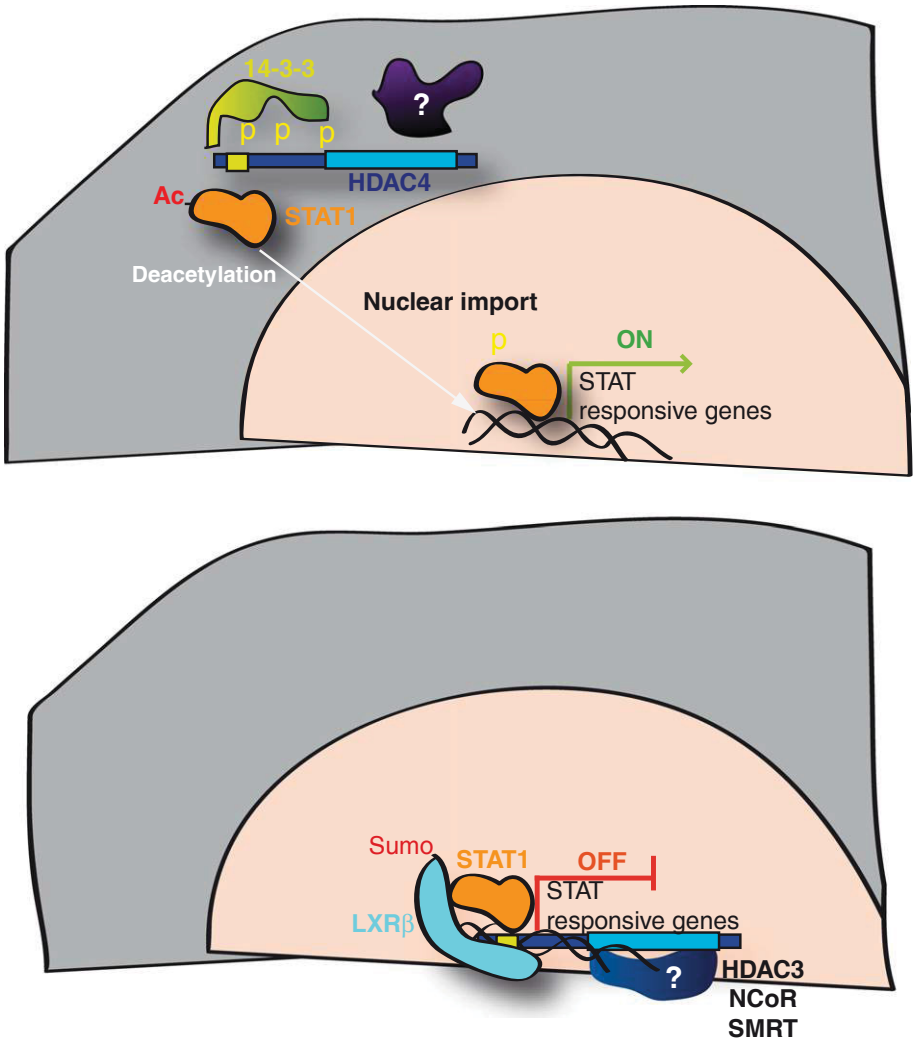

Fig. 2. HDAC4 and STAT1. In the cytoplasm HDAC4 can complex with and deacetylate STAT1 thus favoring nuclear entry and activation of the STAT1 transcriptional program. In the nucleus, when in complex with LRXβ, HDAC4 represses STAT1 transcription by promoting sumoylation. The question marks underline a putative factor supporting the HDAC4 enzymatic activity in the cytoplasm.

also in relation to the other HDAC4 partners are important aspects that need to be comprehended. Moreover, it is important to clarify whether some of the observed differences reflect cell lineage specificities or different experimental settings. Nevertheless for the purposes of this review we can hypothesize that HDAC4 is a Janus protein for STAT1. In the nucleus it promotes the suppression of STAT1 [50], while in the cytoplasm it is capable to deacetylate STAT1, thus favoring its nuclear import and activation [47] (Fig. 2).

6. HDAC4 and forkhead box (FOX) proteins

FOX proteins comprise a heterogeneous family of transcriptional regulators characterized by the presence of a 'forkhead' or 'winged helix' domain involved in DNA binding [55]. The 40 FOX genes have been grouped into 19 subfamilies (from FOXA to FOXS). FOX proteins belonging to the different families utilize different "modus operandi" to regulate gene expression. For example, FOXA subfamily members can operate as pioneer factors, capable of opening up chromatin locally. By contrast, FOXP members operate as classic transcription factors, which recruit chromatin-modifying enzymes. Promiscuous FOX proteins also exist as in the case of FOXO, which show properties both as pioneering and as classic transcription factors [55].

6.1. FOXP3

Different research groups have observed functional connections among class IIa HDACs and FOXP3 transcription factor. Initially an interaction between HDAC7/HDAC9 and FOXP3 was documented in regulatory T cells, a subset of T cells with critical functions in maintaining self-tolerance. In this context, HDAC7 is part of a repressive ternary complex with FOXP3 and the histone acetyltransferase TIP60 [56]. FOXP3 plays a key role in the development and function of Treg cells and the association with HDAC7 was antagonized by stimulation of these cells. Importantly, dysfunction of FOXP3 is associated with fatal autoimmune disease in human [57]. Recently, it has been suggested that alterations in the formation of this repressive complex could be responsible for Treg cell insufficiency and enhanced diabetes [58].

Another study has proposed a different role for HDAC9 in Treg cells. Cell activation triggers HDAC9 nuclear export and since HDAC9 can inhibit FOXP3 function, a role as a limiting factor of Treg suppressive activity was proposed for this class IIa member [59,60].

FOXP3 can also act as a breast tumor suppressor [57]. Other authors have supported a rather indirect connection between FOXP3 and HDAC4 in breast cancer cells. Here, induction of FOXP3 is linked to the up-regulation of the cdk inhibitor p21. This up-regulation correlates

with binding of FOXP3 to p21 intron 1 and displacement of HDAC2 and HDAC4 on a competitive basis [61].

6.2. The FOXO–HDAC4 interaction

Further members of the FOX family can be targets of class IIa HDACs. In the liver HDAC4, 5 and 7 can influence FOXO1 and 3 transcriptional activities. This regulation is under hormonal control and influences glucose homeostasis.

In order to deal with a constant energy requirement, multicellular organisms have evolved a fine tuned regulation, dependent on hormones as well as cell autonomous sensors. Glucose blood levels are continuously adjusted and maintained, within a narrow range, by the antagonizing activity of insulin and glucagon. Insulin stimulates glucose uptake from different tissues [62], whereas glucagon maintains glucose levels during fasting by activating the gluconeogenetic program in hepatocytes [63]. Interestingly, glucagon signaling in primary hepatocytes promotes class IIa HDAC nuclear translocation, reducing their phosphorylation [5] and linking these repressors to the modulation of transcription during the fasted state. Surprisingly, the knock down of HDAC4 and HDAC5 attenuated the induction of gluconeogenetic genes like G6PC, PCK1 and FBP1. This behavior was explained by the effect exerted on FOXO transcription factors by class IIa HDACs. This class of transcription factors supervises several aspects of cell metabolism [64] and in particular they are linked to the regulation of gluconeogenesis [5,65]. The recruitment of HDAC4 to the promoters of gluconeogenetic genes does not affect histones acetylation, but dramatically modulates acetylation of FOXOs. Acetylation on different lys residues of these transcription factors causes a decrease in DNA binding activity [32]. The association between HDAC4 and FOXO1 reduces its acetylation and consequently enhances the transactivating ability. In this case HDAC4 seems to act as a scaffold recruiting HDAC3 [5] that catalyzes removal of the acetyl groups (Fig. 3). Additional mechanisms however cannot be excluded, such as competition for binding proteins regulating FOXO functions, like FCOR, or CBP that promote acetylation [66,67].

While glucagon favors HDAC4 nuclear translocation, insulin promotes its nuclear export [68]. Initially observed in *Drosophila* and then proved also in murine cells, these hormones underpin the classical route of class IIa HDAC regulation: shuttling by phosphorylation. In contrast to MEF2, triggering class IIa HDAC nuclear export leads to FOXO inactivation. The nuclear export of HDAC4 is dependent on SIK2 activity, the ortholog of *Drosophila* SIK3, a kinase that is stimulated by AKT (Fig. 3).

These results are in agreement with initial observations characterizing the regulation of lipogenesis, a metabolic process that generates fatty acids, which is down-regulated during fasting [69]. In this scenario HDAC9 was reported to be associated with the promoters of genes stimulating lipogenesis, like FAS, through the binding of the transcription factor USF1. HDAC9–USF1 interaction affects the acetylation status of this transcription factor and diminishes its transactivating ability. Insulin signaling was described to block the recruitment of HDAC9 on chromatin, thus liberating USF1. Unfortunately these studies do not tackle the biochemical detail behind this interaction.

7. Class IIa HDACs and the muscle adaptation: going conventional

Class IIa HDACs can influence transcriptional programs that modulate skeletal muscle remodeling, according to the functional demands. This adaptive response interests also the metabolic properties with impacts on whole body homeostasis. In this scenario the MEF2–HDAC axis plays again a role as protagonist.

HDAC4, HDAC5 and HDAC9 are abundantly expressed in fast twitch muscles, characterized by a glycolytic metabolism [70]. Exercise stimulation in these types of muscles is accompanied by a reduction of class IIa HDAC levels, achieved through their UPS-mediated degradation. This down-regulation is associated to an enhancement of the oxidative capacity, a behavior explained by the augmented MEF2 activity, which may also increase the insulin-mediated glucose uptake [71]. A role of HDAC4 as a limiting factor of the slow-type muscle program can also be evoked during development [72].

In opposition to training, forced immobilization or pathological conditions like cancer, diabetes or neurological disorders negatively affect muscle mass and significantly modify muscle cell performance and metabolism [73–75]. In denervated mouse muscles, a common experimental model of immobilization, fast twitch tibialis anterioris or gastrocnemius manifests a dramatic augmentation of HDAC4 levels [76,77]. Increased HDAC4 is responsible for the repression of glycolytic genes and for the fiber switch from MHC IIb to the IIa phenotype. This is achieved by the binding of MEF2-sites on promoters of glycolytic genes such as MSE and PFK. This repressive wave is specific since myogenin, another MEF2 target is instead up-regulated during denervation, despite the presence of high HDAC4 levels [76]. This highly selective modulation suggests that the recruitment of HDAC4 to MEF2 promoters is context dependent (and also twitch type dependent) and probably influenced by the presence of additional factors on chromatin [12]. The context dependent influence of MEF2 on its target genes could explain the apparent contradiction between the two model systems (exercise and denervation) in terms of the final outcome (repression or activation) of some of these targets.

HDAC4 in denervated muscle cells can also influence additional responses by binding "unconventional effectors". HDAC4 stimulates the expression of inflammatory molecules like IL-6 and IL-1β [78] that are involved in cancer cachexia. The increase in these cytokines depends on the activation of AP-1 transcription factors. The genetic program leading to muscle remodeling is accomplished by the AP-1 mediated up-regulation of different E3 ligases, like MURF1 and atrogin-1, responsible for muscle atrophy [78,79]. Interestingly, atrogin-1 expression is under the control of FOXO3 as well [80], hence a contribution of HDAC4 through the modulation of this unconventional partner cannot be excluded.

AP-1 induction stems from the prevalent activation of MEKK2 mediated through the control of Lys385 acetylation in the catalytic domain [81]. HDAC4 associating with this kinase reduces its acetylation and increases its kinase activity. Although authors suggest an HDAC4 dependent deacetylation mechanism it should be noted that the used catalytically defective mutant of HDAC4 contains two mutations, one of which abrogates the binding to SMRT/NCoR, therefore blocking the recruitment of HDAC3. Interestingly HDAC4 but not HDAC5 is able to promote AP-1 activity, suggesting a certain degree of specificity.

8. Are class IIa HDACs lysine deacetylases?

A final point that we would like to briefly address is the unresolved issue of the class IIa enzymatic activity, which emerged together with these unconventional partners. Detailed biochemical and structural studies have attributed the deacetylase activity of class II HDACs, to the NCoR–SMRT/HDAC3 complex [9,10,23,82].

HDAC3 acquires enzymatic activity when in complex with NCoR–SMRT, while the dimer HDAC4/NCoR–SMRT requires HDAC3 to become active [23]. Moreover, the addition of HDAC4 to HDAC3/NCoR–SMRT has no additive effect in terms of enzymatic activity [10]. Point mutations in HDAC4 that destroy its binding to HDAC3 determine the complete loss of deacetylase activity [10]. Structural studies have proved that the architecture of the active site of class II HDACs seems to be extremely flexible and dependent on the position of the central Zn ion [82,83]. The enzymatic ineptitude of class II HDACs in vertebrates is due to a Tyr→His substitution in the catalytic site, which impairs the stabilization of the transition state during the reaction [9,82]. The use of trifluoroacetylated (TFA) lysine instead of an acetylated lysine, as substrate allows the re-emergence of a class IIa HDAC enzymatic activity [7]. The observation that the gain of function mutant of HDAC4 (His→Tyr) represses MEF2 reporter likewise the WT [9] indicates that a putative HDAC4 enzymatic

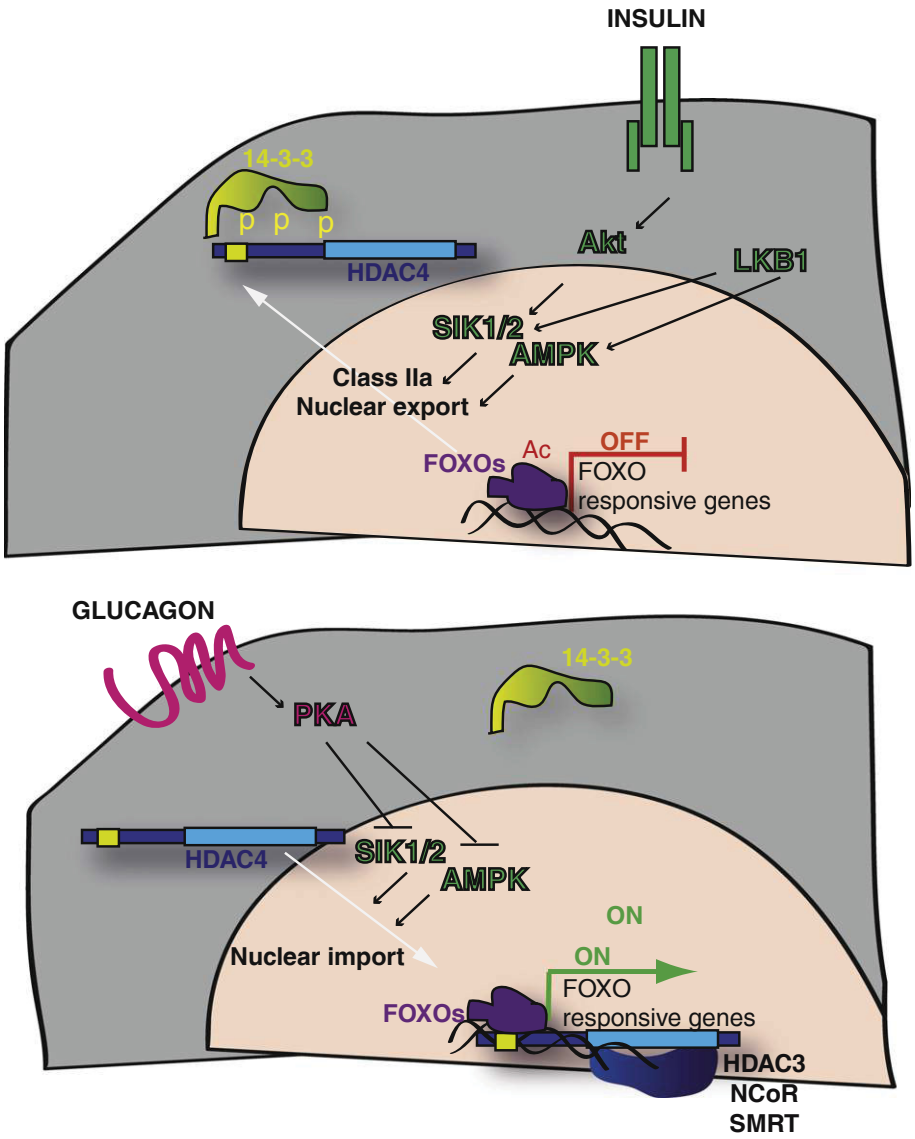

Fig. 3. HDAC4 and FOXOs. In the model proposed by Mihaylova and co-authors [5] for the liver, insulin signaling or metformin (through LKB1) triggers phosphorylation, 14-3-3 binding and nuclear export of HDAC4. Under these conditions FOXOs can be acetylated and inhibited. On the opposite, glucagon, by inhibiting class IIa HDAC kinases (through PKA), favors HDAC4 nuclear accumulation and activation of FOXO-dependent transcription, after its deacetylation. In the FOXOs' circumstance, nuclear accumulation of class IIa HDACs is coupled to transcriptional activation.

activity is completely dispensable for the control of MEF2-dependent transcription [17].

On the other side recent works accredit a deacetylase activity to HDAC4 in the cytoplasm. As discussed above, HDAC4 can deacetylate HIF-1α [30,32], MEKK2 [78] and STAT1 [47] in the cytoplasm. Although impossible to exclude, this event is hardly attributable to the, largely nuclear, class I HDACs. The expression of the His/Tyr mutant of HDAC4 in these models should potentiate the reaction and could help to answer the thorny question: are class II HDACs real enzymes or could they open the hunt for a new cytoplasmic partner of HDAC4?

9. Conclusions

In conclusion a comparative analysis of the available data about the most characterized unconventional partners of HDAC4 and of class IIa has revealed sometimes apparently contradicting results. A surprising observation concerns the possibility of these well-known transcriptional repressors to act also as transcriptional activators, as discussed for FOXOs, HIF or STAT1. It is important to underline that sometimes inconsistencies are present, which could reflect the diverse experimental models and the different experimental approaches. These limitations are traits of a research that is still in its infancy. We are confident that thanks to the additional work and a detailed characterization of the molecular mechanisms driving these interactions, it will be possible to decipher more accurately, the relationships between class IIa HDACs and their unconventional partners.

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Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of ER$^+$ breast tumors

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ABSTRACT **MEF2s transcription factors and class IIa HDACs compose a fundamental axis for several differentiation pathways. Functional relationships between this axis and cancer are largely unexplored. We have found that class IIa HDACs are heterogeneously expressed and display redundant activities in breast cancer cells. Applying gene set enrichment analysis to compare the expression profile of a list of putative MEF2 target genes, we have discovered a correlation between the down-regulation of the MEF2 signature and the aggressiveness of ER$^+$ breast tumors. Kaplan-Meier analysis in ER$^+$ breast tumors evidenced an association between increased class IIa HDACs expression and reduced survival. The important role of the MEF2-HDAC axis in ER$^+$ breast cancer was confirmed in cultured cells. MCF7 ER$^+$ cells were susceptible to silencing of class IIa HDACs in terms of both MEF2-dependent transcription and apoptosis. Conversely, in ER$^-$ MDA-MB-231 cells, the repressive influence of class IIa HDACs was dispensable. Similarly, a class IIa HDAC-specific inhibitor preferentially promoted the up-regulation of several MEF2 target genes and apoptosis in ER$^+$ cell lines. The prosurvival function of class IIa HDACs could be explained by the repression of NR4A1/Nur77, a proapoptotic MEF2 target. In summary, our studies underscore a contribution of class IIa HDACs to aggressiveness of ER$^+$ tumors.—Clocchiatti, A., Di Giorgio, E., Ingrao, S., Meyer-Almes, F.-J., Tripodo, C., Brancolini, C. Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of ER$^+$ breast tumors. *FASEB J.* 27, 942–954 (2013). www.fasebj.org**

Key Words: KLF2 · proliferation · Nur77 · apoptosis · GSEA

CLASS IIA HISTONE DEACETYLASES (HDACs; HDAC4, HDAC5, HDAC7, and HDAC9) are characterized by homology with the yeast enzyme Hda1, tissue-specific expression/functions, and nuclear-cytoplasmic shuttling (1). Cytoplasmic localization of class IIa is coupled to transcriptional activation, while nuclear accumulation promotes transcriptional repression (2). Nuclear-cytoplasmic shuttling is monitored by various kinases that can phosphorylate class IIa HDACs at 14-3-3 binding sites (3, 4). Other post-translational modifications, such as: sumoylation, selective proteolysis, and poly-ubiquitination, keep class IIa HDACs activities in check (5, 6). In addition, their expression is also subjected to transcriptional and translational control (7, 8). Various transcription factors have been reported to interact with class IIa HDACs. The most important are members of the myocyte enhancer factor 2 (MEF2) family (9). Several studies have certified the key role played by the MEF2-HDAC axis during differentiation (10, 11). By contrast, tumor-associated alterations of the axis have been observed only recently (12–14).

Breast cancer comprises a heterogeneous group of diseases characterized by distinct molecular aberrations (15). Sequencing of protein-coding genes has revealed statistically significant mutations of HDAC4 in breast cancer (16). Other studies have reported altered expression of MEF2 members and of HDAC4 (17, 18). Despite some available clues, the contribution of the MEF2-HDAC axis to breast cancer is largely unexplored. In this study, we have investigated the status of the axis in breast cancer cell lines and tumors. Overall, our data imply that targeting class IIa HDACs could represent an interesting therapeutic strategy for impairing proliferation of estrogen receptor-positive (ER$^+$) aggressive tumors.

Abbreviations: AMPK, AMP-activated protein kinase; BrdU, bromodeoxyuridine; ER, estrogen receptor; GSEA, gene set enrichment analysis; HDAC, histone deacetylase; HDI, histone deacetylase inhibitor; KLF2, Krüppel-like factor 2; MEF2, myocyte enhancer factor 2

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MATERIALS AND METHODS

Cell culture, infections, and siRNA transfection

MCF-10A cells were grown as described previously (6). Breast cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS plus penicillin/streptomycin and L-glutamine, with the exception of ZR-75-1 and HCC1937 cells, which were grown in RPMI 1640. The CRM1 inhibitor, leptomycin-B (LC Laboratories, Woburn, MA, USA), was used at 5 ng/ml. AICAR (Sigma-Aldrich, St. Louis, MO, USA) was used. IC_{50} values were obtained using the Rezazurin test. MCF7 and MDA-MB-231 cells expressing GFP or HDAC4-GFP transgenes were generated by retroviral infection as described previously (6). Cells were transfected 24 h after plating by adding the OptiMem medium containing Lipofectamine plus the stealth RNAi oligos (Invitrogen, Carlsbad, CA, USA). Cells were collected after 48 h from transfection.

Immunohistochemistry

Sections of breast tissue, 4 μm thick, were deparaffinized and rehydrated. Subsequently, the slides were microwave-treated in citrate buffer (pH 6; DakoCytomation, Glostrup, Denmark). After neutralization of the endogenous peroxidase, sections were first incubated with protein block Novocastra (UK) for 10 min and next with the anti-human HDAC4 (dilution 1:100). Incubation time was overnight at 4°C. Normal mouse serum was used as negative control. Staining was performed by streptavidin-Hrp/biotyn detection system (LSAB+ System-Hrp; Dako). After counterstaining with hematoxylin (Novocastra, Newcastle upon Tyne, UK), sections were viewed under a Leica DM3000 optical microscope (Leica Microsystems, Wetzlar, Germany), and captions were collected using a Leica DFC320 digital camera (Leica).

Immunoblotting, immunoprecipitation, and immunofluorescence

Immunoblotting was performed as described previously (6). Antibodies used in this work were anti: HDAC3, HDAC5 and HDAC7 (Cell Signaling Technology, Danvers, MA, USA), HDAC9 (Abcam, Cambridge, UK), MEF2A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MEF2C (Cell Signaling Technology), MEF2D and Ran (BD Biosciences, San Jose, CA, USA), EFGR (Santa Cruz Biotechnology), and Bcl-2 (Sigma-Aldrich). For immunoprecipitations, cells were collected directly from culture dishes with a rubber scraper into low-salt lysis buffer (20 mM TrisHCl, pH 7.5; 2 mM EDTA; 10 mM $MgCl_2$; 10 mM KCl; and 1% Triton-X100) supplemented with protease inhibitors. Lysates were incubated with antibody against HDAC4. After incubation with protein A beads (GE Healthcare, Little Chalfont, UK), washes were performed with lysis buffer. For the deacetylase assay, beads were resuspended in the assay buffer (50 mM TrisCl, pH 8; 137 mM NaCl; 2.7 mM KCl; and 1 mM $MgCl_2$) and incubated with Fluor-de-Lys Green Substrate (Enzo Life Sciences, Farmingdale, NY, USA), which comprises an acetylated lysine side chain, for 30 min at 37°C. Deacetylation of the substrate sensitizes it so that treatment with the developer produces a fluorophore. When added to the assay buffer, TSA was at 40 μM final concentration. For immunofluorescence, cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton-X100 in PBS. Next, coverslips were incubated with primary antibodies anti-HDAC4, anti-HMGB1 (Abcam), anti-SMAC (6, 19), and anti-DRP-1 (BD Biosciences). Finally, they were washed twice with PBS and incubated with 488-Alexa or 546-Alexa conjugated secondary antibodies (Invitrogen) and TRITC-phalloidin (Sigma-Aldrich). Cells were examined with a Leica SP confocal microscope.

RNA extraction, retrotranscription reaction, and quantitative PCR

Cells were harvested, and RNA was obtained using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). MMLV reverse transcriptase (Life Technologies) was used for retrotranscription, utilizing 1 μg of total RNA for reaction. qRT-PCR was performed using CFX96 (Bio-Rad, Hercules, CA, USA) and SYBR green technology (Kapa Biosystems, Woburn, MA, USA). Data were analyzed with the $\Delta\Delta C_t$ method, using the geometric mean of *HPRT* and β-*actin* for normalization. Data, from ≥3 independent experiments, were expressed as means ± SE and analyzed with Student's *t* test. qRT-PCR data with the inhibitor were obtained using the geometric mean of *HPRT*, β-*actin*, and *GAPDH* for normalization. All reactions were done in triplicate.

Cell cycle analysis

DNA staining was performed as described previously (6). For S-phase analysis, cells were grown for 3 h with 100 μM bromodeoxyuridine (BrdU). After fixation, coverslips were treated with 1 N HCl (10 min, in ice), followed by 20 min with 2 N HCl at room temperature. Mouse anti-BrdU (Sigma-Aldrich) was used as primary antibody. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich).

Genomic DNA isolation and DNA sequencing

Genomic DNA was isolated and purified using the Qiagen kit (Qiagen, Valencia, CA, USA). PCRs were made using primers covering the different exons. All PCR products were sequenced with the Big Dye Terminator Sequencing RR-100 kit on the ABI PRISM 310 Genetic Analyzer platform (Applied Biosystems, Foster City, CA, USA) on both strands.

Chromatography

Cells were resuspended in the lysis buffer (50 mM TrisHCl, pH7.5; EDTA 0.5 mM; 120 mM NaCl; and Nonidet P-40 0,5%). After centrifugation at 12,000 rpm for 10 min, the extracts were loaded on a column packed with Superose 6 (GE Healthcare). As running buffer, 50 mM TrisHCl (pH 7.5), EDTA 0.5 mM, 120 mM NaCl, and Nonidet P-40 0.1% was used.

Gene set enrichment analysis (GSEA)

Analyses were performed using the GSEA software (http://www.broadinstitute.org/gsea/index.jsp). The list of putative MEF2 target genes was obtained from the Molecular Signature Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). At least 1000 permutations were performed using the "genes_set" permutation type for data obtained from cell lines or "phenotype " permutation type for data obtained for human tumors. Datasets for human tumors were taken from the U.S. National Center for Biotechnology Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). For cell lines, datasets of Mori GSE15026 (20) and Varma GSE32474 (21) were used. For human tumor samples, datasets of Desmedt GSE7390 (22) and Pawitan GSE1456 (23) were employed.

TCGA Kaplan-Meier analysis

Class IIa HDAC expression data were retrieved from the cBio Cancer Genomics Portal (http://www.cbioportal.org/public-portal/). Patients were subdivided into 2 groups: the first consisted of patients with increased expression of at least one member of the family (Z score >2), and with the remnant members having a Z score between -2 and $+2$. In patients that composed the second group, all class IIa HDAC members showed a Z score between -2 and $+2$. All ER^+ tumor samples were taken from the PAM50 Luminal gene expression signatures.

RESULTS

Expression levels of the different components of the MEF2/HDAC axis in breast cancer cell lines

To comprehend the role of the MEF2-HDAC axis in breast cancer, we investigated the expression of the different components of the axis, in breast cancer cell lines and in the nontransformed mammary epithelial cell line, MCF-10A (**Fig. 1*A–C***). The selected cell lines recapitulate genetic alterations commonly observed in breast cancer (Supplemental Table S1). Class IIa HDACs are heterogeneously expressed, and an association between class IIa levels and a breast cancer cell type cannot be evoked. HDAC5 and HDAC9 show the highest expression in luminal cell line T47D. By contrast, HDAC4 levels are elevated in the basal MDA-MB series. Also, HDAC7 is highly expressed in the triple-negative cells, with a peak in the *BRCA1*-mutated HCC1937 cells, where HDAC4 is almost undetectable (Fig. 1*A–C*). MEF2 transcription factors present a more homogenous pattern of expression. MEF2C is expressed at similar levels in all tested breast cancer cell lines, whereas MEF2A and MEF2D expression show some complementarity, well evident in SK-BR-3 and MCF-10A cells.

Immunofluorescence analysis was used to define the subcellular localization of HDAC4. We focused our attention on this deacetylase because it is highly expressed in several basal cell lines, and mutations have been reported in breast cancer (16). To evaluate HDAC4 nuclear-cytoplasmic shuttling, cells were also treated with leptomycin B, an inhibitor of nuclear export. HDAC4 positivity was validated by siRNA transfection (data not shown). In almost all cancer cell lines, HDAC4 shows a diffuse nuclear-cytoplasmic (pancellular) or cytoplasmic localization (Fig. 1*D*). Suppression of nuclear export rapidly promoted its nuclear accumulation, with the exception of MDA-MB-468 cells (Fig. 1*D*). Figure 1*E* exemplifies the analysis performed, showing the data for the luminal cell line MCF7 and the basal cell line MDA-MB-468. These results indicate that in all the tested cell lines, HDAC4 shuttles continuously between the nucleus and cytoplasm, and in MDA-MB-468 cells, there is a defect in its nuclear import.

To evaluate mutations of HDAC4 in the investigated breast cancer cell lines, we sequenced its coding region, from exon 2 to exon 27. Homozygous variations in the HDAC4 coding sequence were found in HCC1937, ZR-75-1, SK-BR-3 and the three MDA-MB cell lines (Supplemental Table S2). However, only in HCC1937 cells was a missense mutation (A786T in exon 18) found. To evaluate HDAC4 levels in breast tumors, immunohistochemistry analysis was performed on 21 cases (Supplemental Fig. S1 and Supplemental Table S3). We evaluated the intensity of HDAC4 staining and its subcellular localization. Similar to breast cancer cell lines, HDAC4 levels are highly variable among different breast tumors, without significant correlations with the proliferative rate or the ER and progesterone receptor (PR) status. Likewise, HDAC4 subcellular localization shows profound variations among the different samples but again without evident correlations with the clinical markers.

Class IIa HDACs-repressive influence on MEF2-dependent transcription in breast cancer cell lines

Because multiple alterations (nuclear-cytoplasmic shuttling, expression levels, point mutations) could potentially affect HDAC4 functions, a simple correlation between breast cancer aggressiveness and HDAC4 levels could be misleading. In principle, to be relevant, any alteration affecting HDAC4 in tumors should affect its repressive activity. According to this hypothesis, we decided to use HDAC4-repressive activity as a tool to unveil its correlation with breast cancer. Krüppel-like factor 2 (KLF2) is a well-known transcriptional target of the MEF2-HDAC axis (6, 24). We utilized KLF2 as a marker to estimate HDAC4-repressive influence. For these studies, we selected MCF7 and MDA-MB-231 cell lines as examples of luminal ER^+ and triple-negative cells.

To understand the role of HDAC4 in the regulation of KLF2 expression in breast cancer cells, MCF7 and MDA-MB-231 cells were silenced for HDAC4, and the mRNA level of the MEF2 target gene was measured by qRT-PCR. In both cell lines, KLF2 expression was not significantly affected by the down-regulation of HDAC4 (**Fig. 2*A***). The effectiveness of HDAC4 silencing was also verified by immunoblot (data not shown). Although HDAC4 is abundantly expressed in several breast cancer cell lines, other members of the family are expressed as well. These deacetylases could interact with MEF2s and overcome the down-regulation of HDAC4. Moreover, compensatory mechanisms have been reported when the expression of a single member of this family is silenced (25). Therefore, we investigated whether silencing of HDAC4 elicited the up-regulation of HDAC5, HDAC7 and HDAC9. As shown in Fig. 2*B*, in MCF7 cells, silencing of HDAC4 triggered the up-regulation of HDAC5, but not of HDAC7 and HDAC9. This response was not observed in MDA-MB-231 cells.

To investigate the role of class IIa HDACs, we decided to silence simultaneously at least 3 members. We focused our attention on HDAC4, HDAC5, and HDAC9,

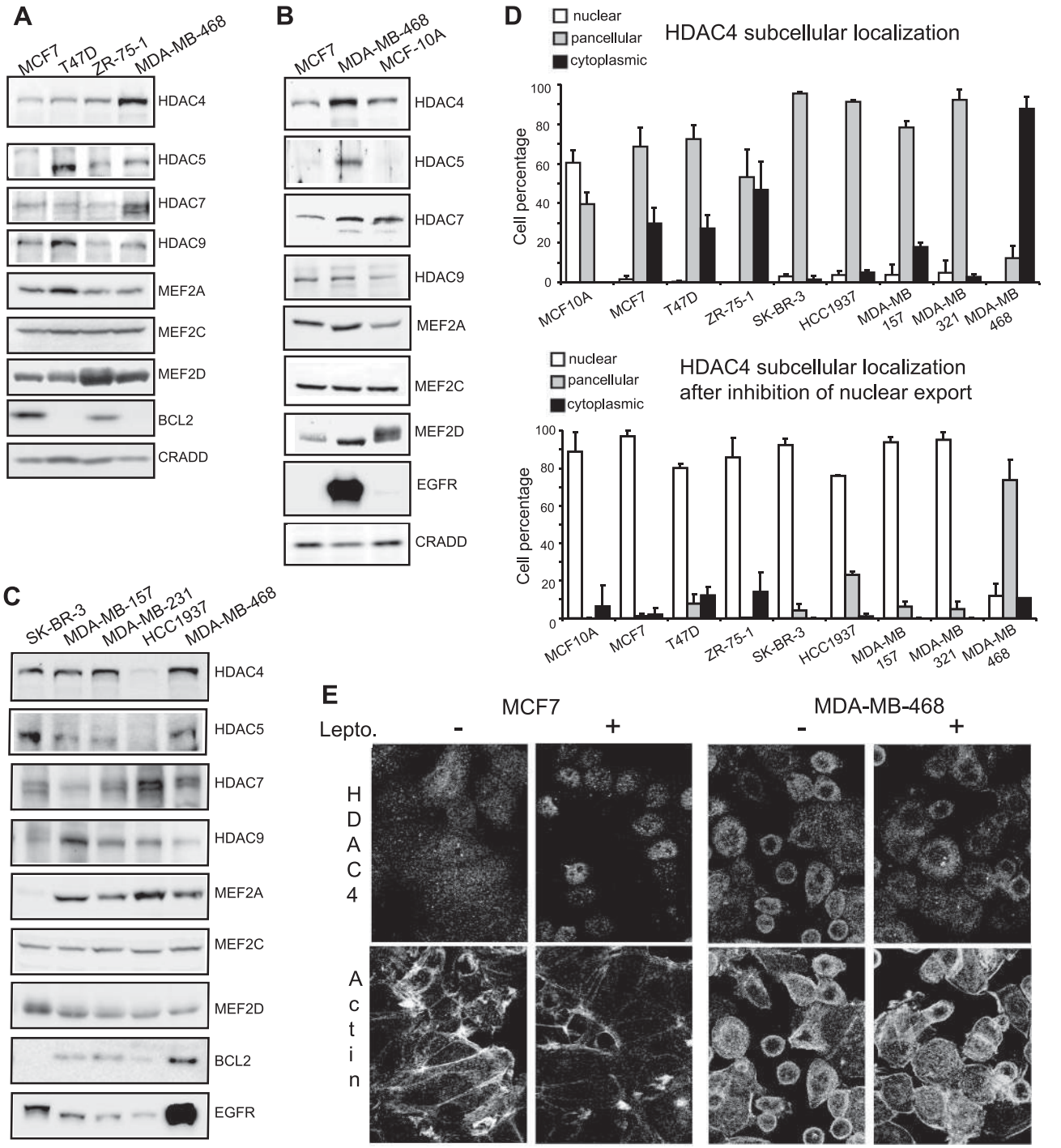

Figure 1. Analysis of HDAC class IIa expression in breast cancer cell lines. *A*) Cellular lysates of indicated breast cancer cell lines were subjected to immunoblot analysis using the specific antibodies. CRADD was used as loading control. *B*) Cellular lysates of indicated cell lines were subjected to immunoblot analysis using the specific antibodies. CRADD was used as loading control. *C*) Cellular lysates of the indicated breast cancer cell lines were subjected to immunoblot analysis using the specific antibodies. *D*) Quantitative analysis of HDAC4 subcellular localization in the indicated cell lines. Immunofluorescence analyses were performed as described in Materials and Methods to visualize HDAC4. When used, leptomycin B was added for 1 h. Approximately 300 cells, from 3 independent experiments, were scored. Data represent arithmetic means ± SD. *E*) Confocal pictures exemplifying the subcellular localization of HDAC4. Leptomycin B was added for 1 h as indicated. Immunofluorescence analysis was performed to visualize HDAC4 subcellular localization. TRITC-phalloidin was used to decorate actin filaments.

because they are phylogenetically closer. The effectiveness of the different siRNAs was also verified by immunoblot (data not shown). KLF2 mRNA was still unperturbed in MDA-MB-231 cells with down-regulated HDAC4, HDAC5, and HDAC9. By contrast, in MCF7 cells, the expression of the MEF2 target, KLF2, was

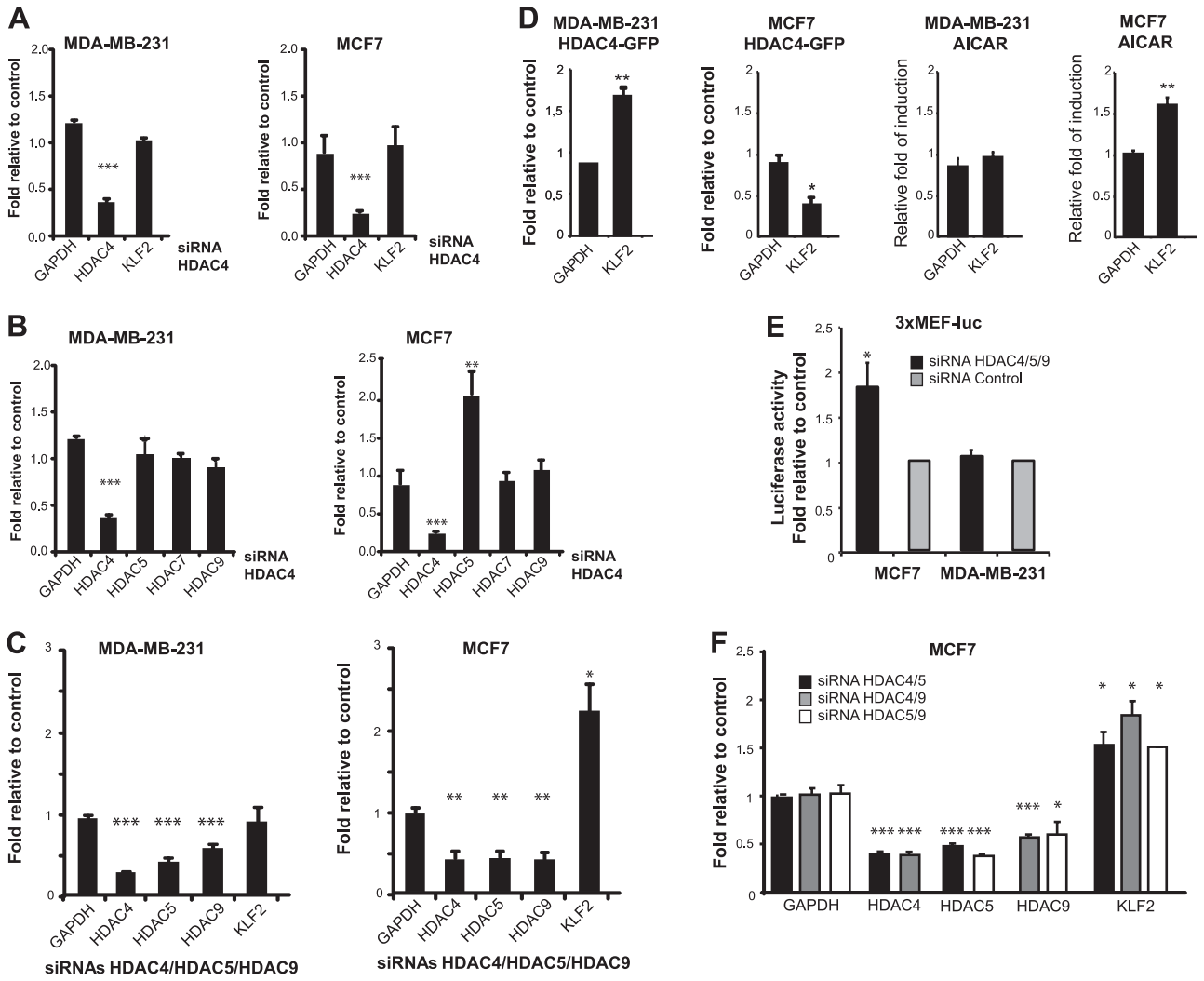

Figure 2. Regulation of KLF2 expression by class IIa HDACs in breast cancer cells. *A*) qRT-PCR analysis was performed to quantify mRNA levels of the MEF2 target gene *KLF2* and of *HDAC4* to verify silencing efficiency. *GAPDH* was used as control gene. MCF7 and MDA-MB-231 cells transfected with siRNA against HDAC4 were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. *B*) qRT-PCR analysis was performed to quantify mRNA levels of *HDAC4*, *HDAC5*, *HDAC7*, and *HDAC9*. *GAPDH* was used as control gene. MCF7 and MDA-MB-231 cells transfected with siRNA against HDAC4 were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. *C*) qRT-PCR analysis was performed to quantify mRNA levels of the MEF2 target gene *KLF2* and of *HDAC4*, *HDAC5*, and *HDAC9* to verify silencing efficiency. *GAPDH* was used as control gene. MDA-MB-231 and MCF7 cells cotransfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the same amount of a control siRNA were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. *D*) MCF7 and MDA-MB-231 cells expressing HDAC4-GFP or GFP were lysed, and mRNAs were extracted. qRT-PCR analysis was performed to quantify mRNA levels of *KLF2*. Fold induction was calculated as the ratio relative to GFP-tramsfected cells. MCF7 and MDA-MB-231 cells were treated with AICAR (200 μM) for 24 h. qRT-PCR analysis was performed to quantify mRNA levels of *KLF2*. *GAPDH* was used as control gene. Fold induction was calculated as the ratio relative to untreated cells. *E*) After 24 h from HDAC4, HDAC5, HDAC9, and control silencing, cells were transfected with 3xMEF2-Luc reporter (1 μg) and the internal control luciferase reporter pRL-CMV (20 ng) to normalize the transfection efficiency. Assays were performed 24 h later. *F*) qRT-PCR analysis was performed to quantify mRNA levels of the MEF2 target gene *KLF2*. *GAPDH* was used as control gene. MCF7 cells cotransfected with the indicated combinations of siRNAs against HDAC4, HDAC5, and HDAC9 or with the same amount of a control siRNA were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. Data are from 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

up-regulated (Fig. 2*C*). To verify this result, we generated MCF7 and MDA-MB-231 cells stably expressing HDAC4-GFP or GFP alone. qRT-PCR analysis confirmed that in MCF7 cells, KLF2 is regulated by HDAC4. Surprisingly, KLF2 expression was up-regulated in MDA-MB-231 cells expressing HDAC4-GFP (Fig. 2*D*). However, it should be taken into account that retroviral infection with HDAC4-GFP elicited a strong inhibition of cell growth, as previously observed (26), which results in the selection of few clones positive for HDAC4. Hence, we used an alternative strategy to corroborate the differential requirement of class IIa HDACs, in the two cell lines. To release class IIa HDACs-mediated repression, we

promoted their export through the engagement of the AMP-activated protein kinase (AMPK; ref. 25). MDA-MB-231 and MCF7 cells were treated with the AMPK activator AICAR, and mRNA was isolated for qRT-PCR analysis. KLF2 levels were up-regulated after AICAR treatment only in MCF7 cells. Finally, transcription from a MEF2 artificial promoter was selectively augmented in MCF7 cells silenced for the different HDACs (Fig. 2*E*).

To elucidate which deacetylases are implicated in the repression of KLF2 expression in MCF7 cells, we evaluated the combination of two different siRNAs. As illustrated in Fig. 2*F*, silencing of two HDACs at a time was sufficient to up-regulate KLF2 levels, although less potently compared to the triple siRNA. Individual silencing of HDAC5 or HDAC9 was not sufficient to augment KLF2 mRNA (data not shown). We also reduced repressive influence of the HDAC4 multiprotein complex, and a different transcriptional regulation limited the effeact of class IIa HDACs on KLF2 expression in MDA-MB-231 cells. Class IIa HDACs seem to be dispensable for the control of KLF2 transcription in MDA-MB-231 cells. Hence, in the triple-negative cell line, the MEF2-HDAC axis could be altered. To answer this question, we initially compared the capability of class IIa HDACs to form a complex with MEF2s. Coimmunoprecipitation showed that in both cell lines, HDAC4 can be isolated in a complex with MEF2D (**Fig. 3*A***).

HDAC4 represses transcription by bridging the enzymatically active SMRT/N-CoR-HDAC3 complex to target promoters (27). After fractionation of cellular extracts overexpressing HDAC4 on a superose 6 column, enzymatic activity was found in a high-molecular-weight (HMW) complex with mass $>$ 0.66 MDa (27). Hence, we investigated whether endogenous HDAC4 also could be isolated in an HMW complex and whether differences could be appreciated between the two cell lines. Immunoblotting of the different fractions visualized for HDAC4, MEF2D and HDAC3 are shown in Fig. 3*B*. Overall, the pattern is similar in the two cell lines. Only limited amounts of HDAC4 and MEF2D were visualized in fractions of $>$0.66 MDa. By contrast, HDAC3 was almost entirely found in fractions $>$ 0.66 MDa.

To confirm that MEF2D and HDAC4 can interact in an HMW complex, the different fractions were immunoprecipitated for HDAC4 and visualized for MEF2D. We performed this experiment in MDA-MD-231 cells that express higher amounts of HDAC4. An enrichment of MEF2D in the $>$0.66-MDa complex and a reduction in the low-molecular-weight fractions can be appreciated in Fig. 3*B*. In summary, these studies indicate that in both cell lines, HDAC4 binds MEF2D and can form protein complexes of mass $>$ 0.66 MDa. Theoretically, in both cell lines, HDAC4 should be competent for suppressing MEF2-dependent transcription.

To prove this assumption, we analyzed the deacetylase activity associated with HDAC4. Protein complexes containing HDAC4 were isolated using anti-HDAC4 antibody, and deacetylase activity was scored using an acetyl lysine as substrate. The deacetylase activity associated with HDAC4 was higher in MCF7 compared to MDA-MB-231 cells (Fig. 3*C*). This difference was even more impressive considering that much more HDAC4 was immunoprecipitated from MDA-MB-231 cells (Fig. 3*D*). Normalization of the enzymatic activity, relative to the amount of immunoprecipitated HDAC4, evidenced a 5-fold increase of HDAC4-associated deacetylase activity in MCF7 cells (Fig. 3*E*). Since HDAC3 provides an important contribution to the HDAC4-associated deacetylase activity, we analyzed HDAC3 levels in the two cell lines. HDAC3 levels were reduced in MDA-MB-231 compared to MCF7 cells (Fig. 3*F*).

Taking into account that KLF2 is a target of the MEF2-HDAC axis in breast cancer cells and that class IIa HDAC-repressive influence is reduced in MDA-MB-231 cells, its expression should be elevated in MDA-MB-231 compared to MCF7 cells. qRT-PCR analysis verified that KLF2 expression is almost 6-fold higher in MDA-MD-231 cells (Fig. 3*G*). We also evaluated whether KLF2 is subjected to different regulation in the two cell lines. The PI3K/AKT pathway can regulate KLF2 expression (28). Inhibition of this pathway has different consequences on KLF2 in the two cell lines (Fig. 3*H*). In MCF7 cells, the PI3K inhibitor LY294002 augmented KLF2 levels, whereas in MDA-MB-231 cells, it reduced KLF2 expression. This result indicates that KLF2 expression is under different regulation in the two cell lines.

Class IIa HDACs regulate survival of MCF7 cells

Having proved a repressive influence of class IIa HDACs in MCF7 cells, we decided to explore the contribution of these HDACs to cell proliferation. The simultaneous down-regulation of HDAC4/5/9 significantly affected proliferation in MCF7 but not in MDA-MB-231 cells (**Fig. 4*A***). Conversely, single silencing of HDAC4 was insufficient to reduce proliferation (Supplemental Fig. S2). Cytofluorimetric analysis did not revealed overt changes in cell-cycle profiles of MCF7 cells silenced for HDAC4, HDAC5, and HDAC9 (Fig. 4*B*). Paradoxically, a small rise in cells replicating the DNA was observed after BrdU staining (Fig. 4*C*). Next we evaluated whether class IIa HDACs could restrain apoptosis in MCF7 cells. Trypan blue assay revealed an increase in cell death when HDAC4, HDAC5, and HDAC9 levels were reduced after siRNA transfection (Fig. 4*D*). Apoptosis was confirmed by scoring the release of SMAC from mitochondria, a mitochondrial outer membrane permeabilization marker, and the accumulation of HMGB1 in the cytoplasm (Fig. 4*E*). In both assays, down-regulation of class IIa HDACs significantly increased the percentage of cells showing apoptotic features (Fig. 4*F*).

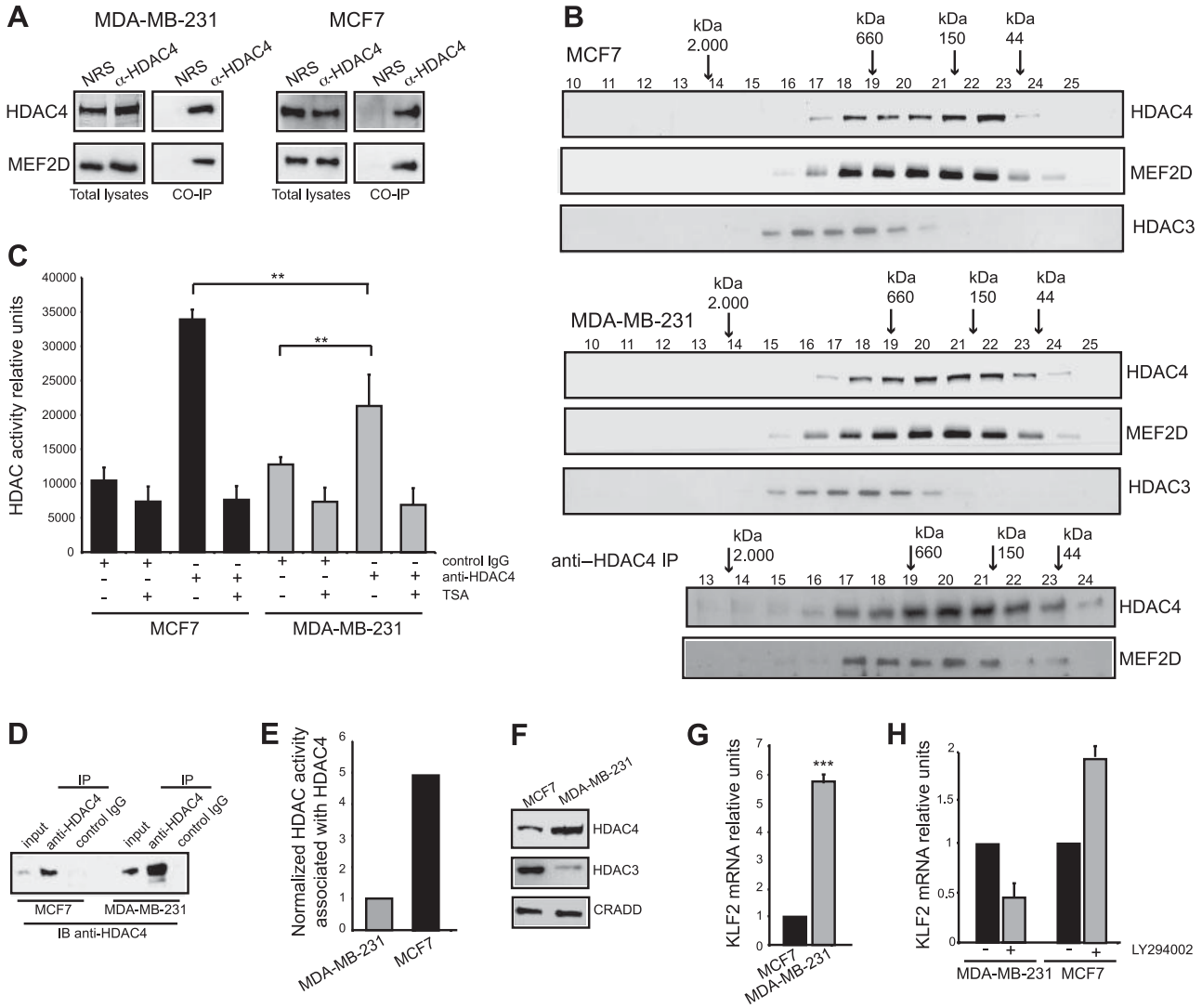

Figure 3. Understanding the differential contribution of class IIa HDACs in ER$^+$ (MCF7) and ER$^-$ (MDA-MB-231) breast cancer cells. *A*) Cellular lysates from MDA-MB-231 and MCF7 cells were immunoprecipitated using an anti-HDAC4 antibody or normal rabbit serum (NRS). Immunocomplexes were next probed with anti-MEF2D or anti-HDAC4 antibodies, as indicated. A fraction of the lysates before immunoprecipitation was used as input (total lysates). *B*) Cellular lysates from MDA-MB-231 and MCF7 cells were separated on a Superose 6 gel-filtration column. Fractions were analyzed for the presence of HDAC4, MEF2D, and HDAC3 by immunoblotting. Next, fractions from MDA-MB-231 cells were immunoprecipitated using the anti-HDAC4 antibody, and immunoblotting was performed with the anti-MEF2D or anti-HDAC4 antibodies. Arrows indicate the elution positions of molecular mas standards. *C*) Cellular lysates from MDA-MB-231 and MCF7 cells were immunoprecipitated using an anti-HDAC4 antibody or control rabbit immunoglobulin (IgG). After several washes, immunocomplexes were incubated with the Fluor de Lys substrate. TSA was used at 40 μM final concentration. Data are from 3 independent experiments. *D*) A fraction of the immunoprecipitations analyzed for the deacetylase activity was separated by SDS-PAGE, and after immunoblotting, HDAC4 was visualized using anti-HDAC4 antibody. *E*) Densitometric analysis was performed on the immunoblot in panel *B* to normalize HDAC activity to the amount of HDAC4 purified from the two cell lines. *F*) Cellular lysates generated from MCF7 and MDA-MB-231 cell lines were subjected to immunoblot analysis using specific antibodies as indicated. CRADD was used as loading control. *G*) qRT-PCR analysis was performed to compare KLF2 mRNA levels between MCF7 and MDA-MB-231 cells. Samples were normalized to *HPRT*, *GAPDH*, and β-*actin*. Fold induction was calculated as the ratio relative to KLF2 mRNA levels in MCF7 cells. Data are from ≥3 independent experiments. *H*) qRT-PCR analysis of KLF2 mRNA levels in MCF7 and MDA-MB-231 cells after treatment for 12 h with LY294002 (5 μM). Data are from ≥3 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

Class IIa HDACs repress the expression of the proapoptotic gene Nur77/NR4A1 in MCF7 cells

Ectopic expression of KLF2 could not trigger apoptosis in MCF7 cells (data not shown). The nuclear orphan receptor Nur77/NR4A1 is another transcriptional target of the MEF2-HDAC complex, and it can elicit apoptosis (29). To gain insight on the prosurvival activity of class IIa HDACs, we analyzed whether the expression of Nur77 family members (Nur77/NR4A1, Nurr1/NR4A2, and NOR1/NR4A3) is repressed by these deacetylases in MCF7 cells. Only the expression of Nur77/NR4A1 was significantly up-regulated when class IIa HDACs were silenced (**Fig. 5*A***).

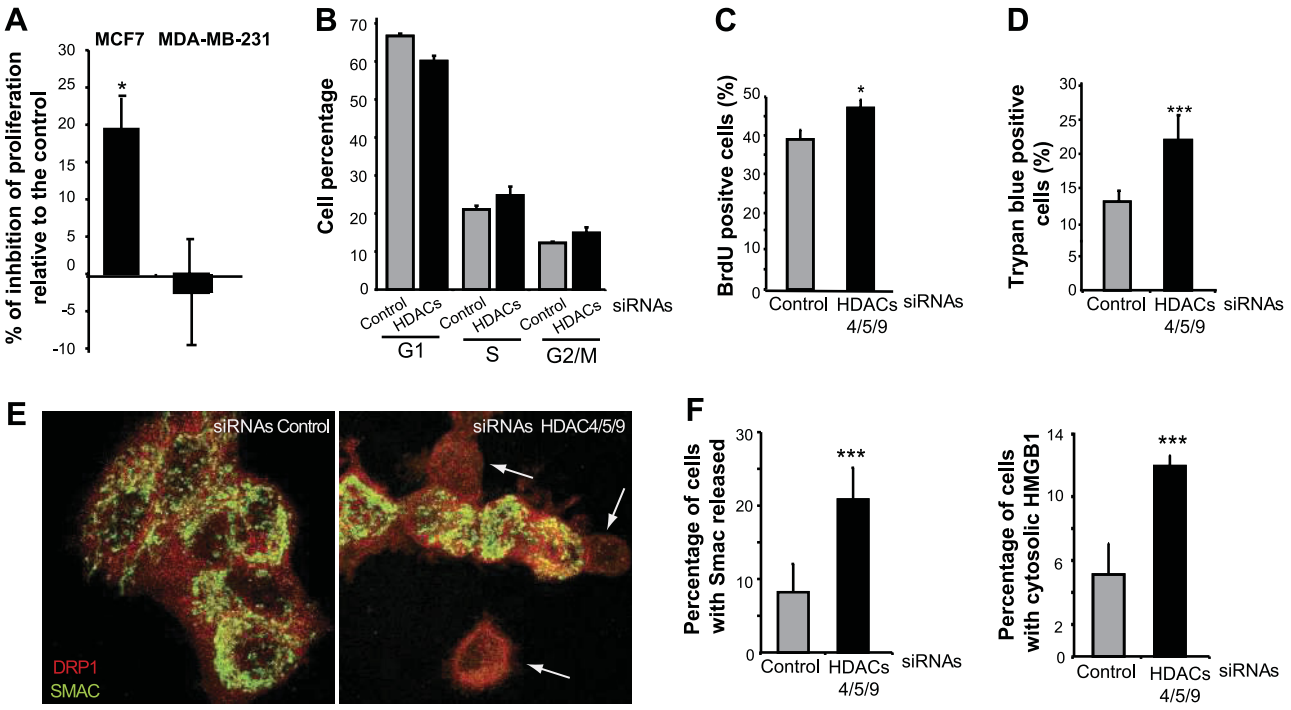

Figure 4. Class IIa HDACs control MCF7 cell survival. *A*) MCF7 and MDA-MB-231 cells were transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the control siRNA. After 48 h, cells were counted. Data are presented as percentage of inhibition of the triple siRNAs relative to control and are expressed as means ± SD. $*P < 0.05$. *B*) MCF7 cells were transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the control siRNA. After 48 h, cells were fixed, and cell cycle profiles were assessed by FACS analysis (means±SD, $n=3$). *C*) MCF7 cells were transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the control siRNA. After 33 h, BrdU was added for 3 h, and then cells were processed for immunofluorescence (means±SD, $n=3$). *D*) MCF7 cells were transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the control siRNA. After 48 h, cell death was analyzed after Trypan blue staining (means±SD, $n=3$). *E*) Confocal images illustrating the subcellular localization of SMAC and HMGB1 in MCF7 cells transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the control siRNA. At 36 h after transfection, cells were fixed and processed for immunofluorescence. TRITC-phalloidin was used to decorate actin filaments and anti-DRP1 antibodies to stain the cytoplasm. Images are shown in pseudocolors. Arrows point to cells with released SMAC or HMGB1. *F*) Quantitative analysis of SMAC and HMGB1 localization as described in panel *E*.

In other cell lineages, HDAC7 plays an important role in the regulation of Nur77/NR4A1 expression. In MCF7 cells, silencing of HDAC7 influenced Nur77 expression only in combination with the silencing of other class IIa HDACs (unpublished results). These results imply that in breast cancer cells, HDAC7 affects Nur77 expression comparably to the other members of the family.

Next, we compared the expression levels of Nur77 family members between MCF7 and MDA-MB-231 cells. Contrary to KLF2, Nur77 levels were dramatically reduced in MDA-MB-231 cells; whereas expression of Nurr1 and NOR1 was equivalent in the two cell lines (Fig. 5*B*). Similar to KLF2, in MDA-MB-231 cells, the expression of Nur77 family members was unaffected by the triple silencing (Fig. 5*C*). Finally, we verified whether enhancing Nur77 levels in MCF7 cells could elicit apoptosis. Nur77s fused to GFP or GFP alone were transiently transfected in MCF7 cells, and apoptosis was evaluated by scoring the release of SMAC from mitochondria. As exemplified by representative immunofluorescence images (Fig. 5*D*) and by quantitative analysis (Fig. 5*E*), expression of Nur77 promoted SMAC release from mitochondria. The increase in apoptosis was confirmed by the elevated rate of procaspase-3 processing in Nur77-overexpressing cells (Fig. 5*F*).

Repression of the MEF2 signature correlates with aggressiveness of ER$^+$ tumors

Our study in breast cancer cell lines suggests that class IIa HDACs could influence MEF2-dependent transcription in ER$^+$ but not in ER$^-$ tumors. To explore this hypothesis, we compared the MEF2-trancriptional signature in different breast tumors. For this aim, we employed a list of genes that carry in their proximal promoter the MEF2-binding site (http://www.broadinstitute.org/gsea/msigdb/index.jsp). We de-

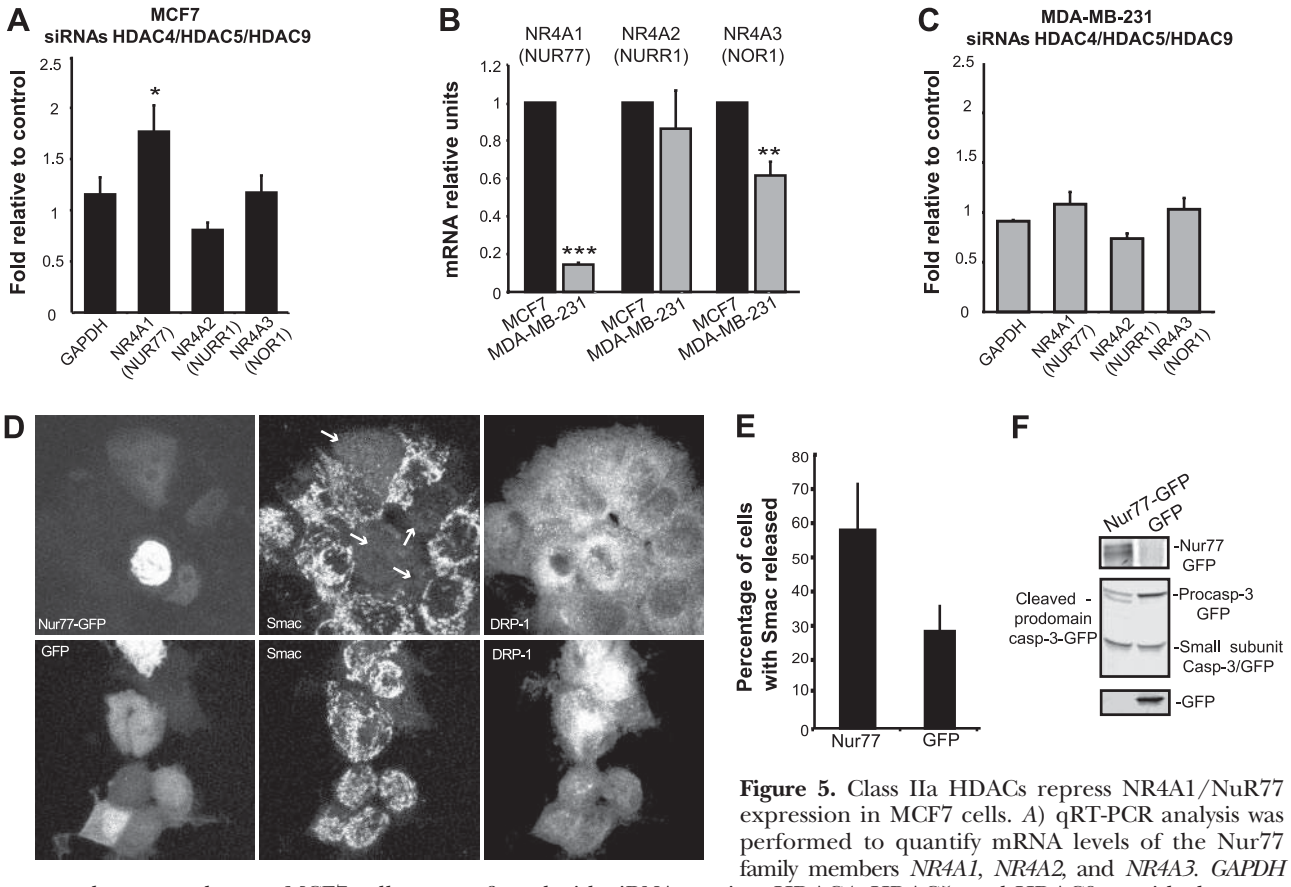

Figure 5. Class IIa HDACs repress NR4A1/NuR77 expression in MCF7 cells. *A*) qRT-PCR analysis was performed to quantify mRNA levels of the Nur77 family members *NR4A1*, *NR4A2*, and *NR4A3*. *GAPDH* was used as control gene. MCF7 cells cotransfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the same amount of a control siRNA were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. *B*) qRT-PCR analysis was performed to compare *NR4A1*, *NR4A2*, and *NR4A3* mRNA levels between MCF7 and MDA-MB-231 cells. Samples were normalized to *HPRT*, *GAPDH*, and β-*actin*. Fold induction was calculated as the ratio relative to *NR4A* mRNA levels in MCF7 cells. *C*) qRT-PCR analysis was performed to quantify mRNA levels of the Nur77 family members *NR4A1*, *NR4A2*, and *NR4A3*. *GAPDH* was used as control gene. MDA-MB-231 cells cotransfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with a control siRNA were lysed, and mRNAs were extracted. Fold induction was calculated as the ratio relative to control siRNA-transfected cells. *D*) Confocal images illustrating the subcellular localization of SMAC in MCF7 cells transfected with NR4A1/Nur77-GFP or with GFP alone. At 24 h after transfection, cells were fixed and processed for immunofluorescence. Anti-DRP1 antibodies were used to stain the cytoplasm. Arrows point to cells with released SMAC. *E*) Quantitative analysis of SMAC localization as described in panel *D*. *F*) Caspase-3/GFP together with Nur77-GFP or GFP alone was transiently expressed in MCF7 cells. After 24 h, cell lysates were generated and subjected to immunoblotting using the anti-GFP antibody. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

cided to exclude from the analysis MEF2 target genes that are modulated by ER (Supplemental Fig. S3). We began by comparing the expression levels of the MEF2 target genes in MCF7 and MDA-MB-231 cells, using GSEA and two different data sets (20, 21). **Figure 6*A*** illustrates a reduction in the expression of the MEF2 target genes in MCF7 cells compared to MDA-MB-231 cells ($P<0.001$ and $P<0.04$), which reflects the behavior of KLF2.

Next, we proved whether this correlation is also maintained in human breast cancers. We initially employed two different data sets comparing poorly differentiated tumors, classified grade 3 (G3) by histological grade, subdivided into ER^+ and ER^-. Figure 6*B* highlights that, similar to MCF7 and MDA-MB-231 cells, a negative correlation appears between G3 ER^+ tumors and the MEF2 signature with respect to ER^- G3 tumors ($P<0.025$ and $P<0.045$), using two different datasets (22, 23).

The down-regulation of the MEF2 signature in G3 ER^+ tumors prompted us to investigate the correlation between the signature and the aggressiveness of ER^+ tumors. When we compared the MEF2-signature in G1 and G2 *vs.* G3 ER^+ breast cancers, a down-regulation was evident using two different datasets (refs. 22, 23 and Fig. 6*C*; $P<0.001$, $P<0.001$, and $P<0.02$). On the contrary, when the analysis was performed between G1 and G2 ER^+ tumors, a differential correlation was not proved (Fig. 6*D*). Similarly, repression of the MEF2 signature was not discerned when G2 and G3 ER^- tumors were compared (Fig. 6*D*).

Targeting class IIa HDACs in ER^+ breast tumors

To validate the MEF2 signature used in the GSEA, we investigated whether modulation of class IIa HDACs could influence the expression of these genes. For this study, we used *N*-lauroyl-(L)-phenylalanine, a recently

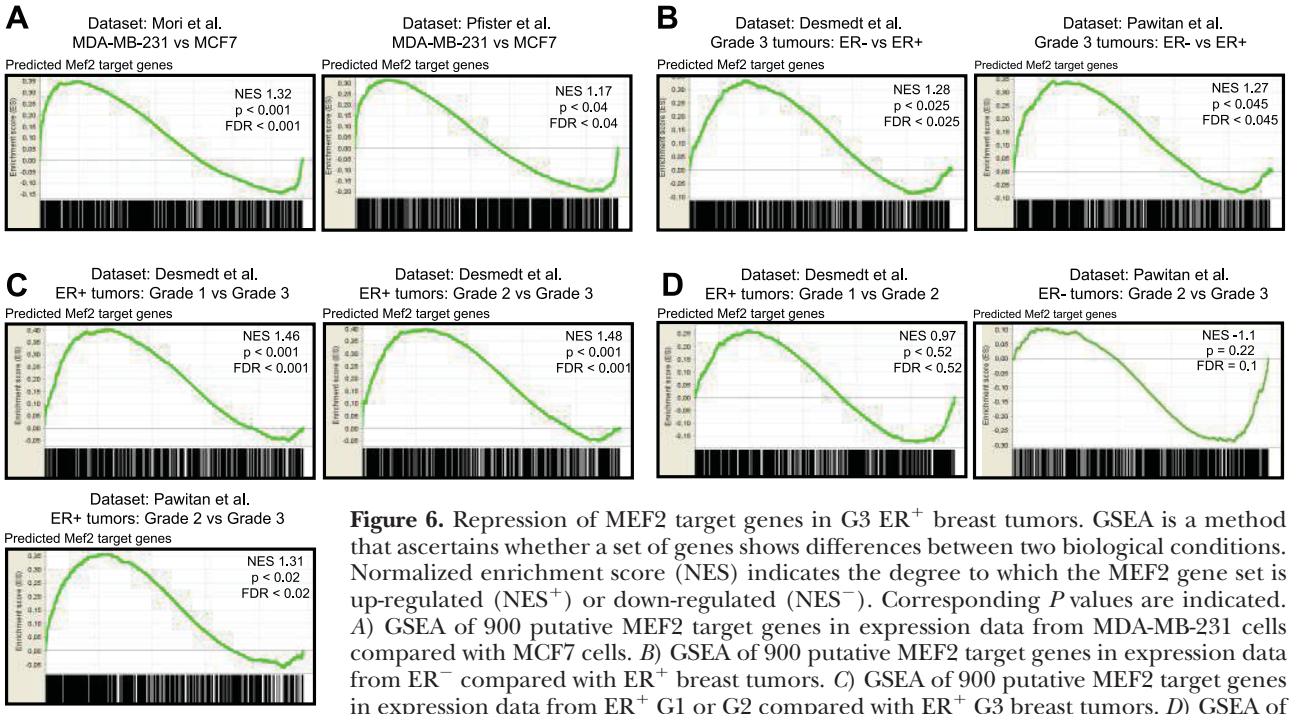

Figure 6. Repression of MEF2 target genes in G3 ER$^+$ breast tumors. GSEA is a method that ascertains whether a set of genes shows differences between two biological conditions. Normalized enrichment score (NES) indicates the degree to which the MEF2 gene set is up-regulated (NES$^+$) or down-regulated (NES$^-$). Corresponding *P* values are indicated. *A*) GSEA of 900 putative MEF2 target genes in expression data from MDA-MB-231 cells compared with MCF7 cells. *B*) GSEA of 900 putative MEF2 target genes in expression data from ER$^-$ compared with ER$^+$ breast tumors. *C*) GSEA of 900 putative MEF2 target genes in expression data from ER$^+$ G1 or G2 compared with ER$^+$ G3 breast tumors. *D*) GSEA of 900 putative MEF2 target genes in expression data from ER$^+$ G1 compared with ER$^+$ G2 or from ER$^-$ G2 compared with ER$^-$ G3 breast tumors.

identified class IIa specific histone deacetylase inhibitor (HDI; ref. 30). The deacetylase activity associated with immunoprecipitated HDAC4, but not with immunoprecipitated HDAC3, was inhibited by the HDI (Supplemental Fig. S4).

When MCF7 and MDA-MB-231 cells were treated with the HDI, proliferation impairment (IC_{50} at 48 h: 230±12 μM) and cell death were observed only MCF7 cells (**Fig. 7*A, B***). As for the siRNA experiments, growth of MDA-MB-231 cells was unaffected by the presence of the HDI. Next, we explored the effect of the class IIa HDI on a panel of MEF2 targets (NR4A1, KL2, KLF3, KLF5, MARK1, GADD45γ, IPO4, PPAP2A, and USP47), genes of the signature used in the GSEA. Figure 7*C* shows that expression of several MEF2 targets was significantly increased (>2-fold) in MCF7 cells treated with the HDI (KLF-2, NR4A1, KLF3, MARK1, and GADD45γ). This up-regulation was less evident in MDA-MB-231 cells. Here, only KLF3 induction mimicked the response observed in MCF7 cells. NR4A1 induction was less prominent, whereas KLF2, MARK1, and GADD45γ were not significantly up-regulated. By contrast, IPO4 induction was observed only in MDA-MB-231 cells. Of note, the HDI was a more potent inducer of the MEF2-dependent transcription with respect to the triple siRNA. The specificity of the antiproliferative effect elicited by the HDI was verified by comparing two compounds structurally resembling specific portions of the inhibitor: L-phenylalanine-methyl ester and dodecanoyl-(D/L)-homoserine. When dose-dependent studies were performed in MCF7 cells using the HDI and the two controls, effect on proliferation, induction of apoptosis, and activation of MEF2-dependent transcription were observed only in response to the class IIa inhibitor (Fig. 7*D–F*).

We also extended this study to other ER$^+$ and ER$^-$ cell lines (Supplemental Fig. S5). Overall, the ER$^-$ cells were resistant to the antiproliferative effect of the HDI and impotent in up-regulating Nur77 expression, whereas the ER$^+$ cell line (ZR-75-1) entered apoptosis and up-regulated Nur77 expression. These results prompted us to analyze the involvement of class IIa HDACs in the aggressiveness of ER$^+$ tumors. To begin to answer this question, we interrogated the Cancer Genome Atlas to find a correlation between survival and the expression levels of class IIa HDACs. The redundant role of class IIa HDACs inspired us to consider the different members of the family as a single entity. Patients with ER$^+$ tumors were subdivided into two groups: high and normal class IIa HDACs (see Materials and Methods). Kaplan-Meier analysis showed that patients with high class IIa HDAC expression had a median survival of 85 mo, compared to patients with low class IIa HDAC expression, who had a median survival of 114 mo (Fig. 7*G*).

DISCUSSION

Breast cancer is a heterogeneous disease in terms of morphological appearance, molecular features, behavior, and response to therapy (24). Our studies indicate that class IIa HDACs are heterogeneously expressed in different subtypes of breast cancer cell lines. This heterogeneity was also confirmed in breast tumors by exploring public-domain databases, such as GEO and

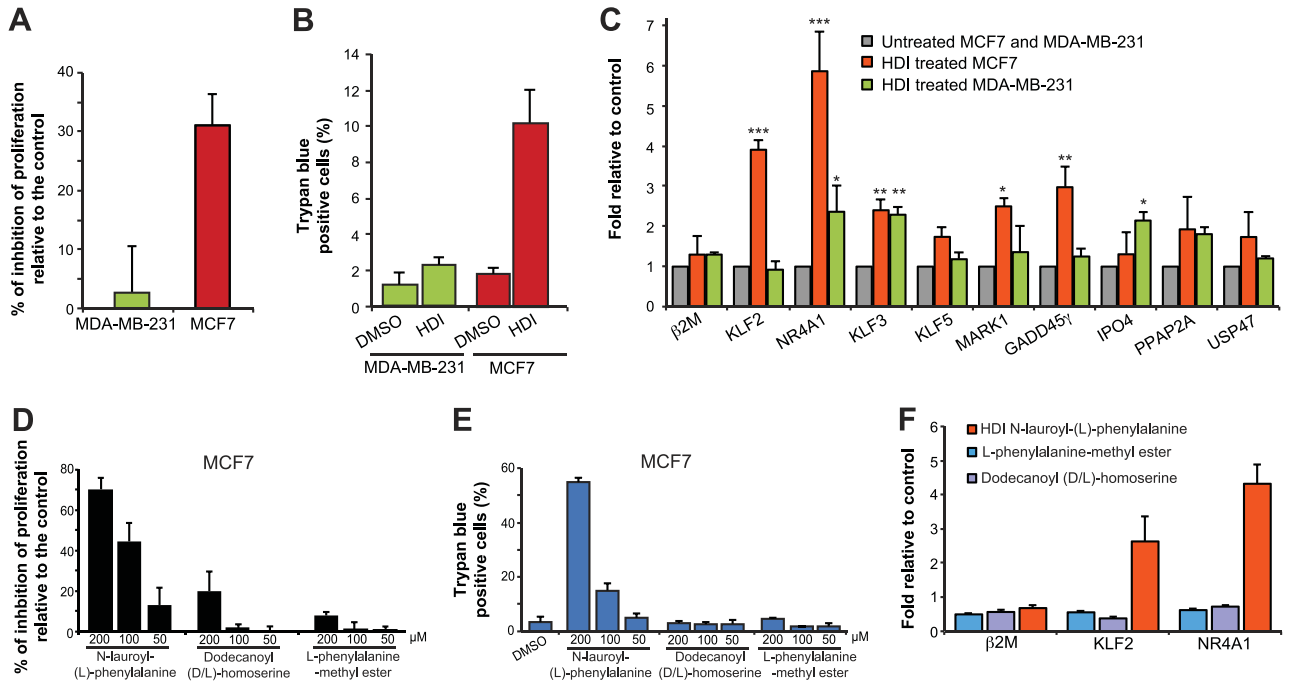

Figure 7. Targeting class IIa HDACs in ER$^+$ breast tumors. *A*) MCF7 and MDA-MB-231 cells were treated with 100 μM of the class IIa HDI *N*-lauroyl-(L)-phenylalanine. After 48 h, cells were counted. Data are presented as percentage of inhibition of the HDI relative to DMSO and are expressed as means ± SD. *B*) MCF7 and MDA-MB-231 cells were treated with HDI (100 μM). After 48 h, cell death was analyzed after Trypan blue staining (means±SD, n=3). *C*) qRT-PCR analysis was performed to evaluate the expression of a panel of MEF2 target genes selected from the set used in the GSEA. MCF7 and MDA-MB-231 cells were treated for 48 h with 100 μM HDI. Fold induction was calculated as the ratio relative to mRNA levels in untreated MCF7 and MDA-MB-231 cells, respectively. β2-Microglobulin was used as control. *D*) MCF7 cells were treated with the indicated concentrations of HDI and with two compounds, structurally resembling specific portions of the inhibitor: L-phenylalanine-methyl ester and dodecanoyl-(D/L)-homoserine. After 48 h, cells were counted. Data are presented as percentage of inhibition of the different compounds relative to DMSO and are expressed as means ± SD; n = 3. *E*) MCF7 cells were treated as in panel *D*. After 48 h, cell death was analyzed after Trypan blue staining (means±SD; n=3). *F*) qRT-PCR analysis was performed to evaluate the expression of the MEF2 target genes KLF2 and NR4A1. MCF7 cells were treated for 48 h with 100 μM of the indicated compounds. Fold induction was calculated as the ratio relative to mRNA levels in untreated MCF7 cells. β2-Microglobulin was used as control. *G*) Kaplan-Meier analysis based on class IIa HDACs expression using data from TCGA ER$^+$ breast cancers. All cases, n = 372; high class IIa HDAC expression cases, n = 29.

Oncomine, and by immunohistochemistry for HDAC4. A second feature of class IIa HDACs was redundancy. Class IIa HDACs act redundantly to suppress MEF2-dependent transcription, and compensatory circuits controlling their levels also exist (25). These characteristics hint that searching for a correlation between expression levels of a single member of the family and a particular breast cancer subtype could be an oversimplistic approach. Hence, we used a different strategy. We measured the contribution of class IIa HDACs to breast cancer indirectly, by ranking the expression levels of a list of putative MEF2 target genes. In this manner we have found a correlation between the down-regulation of several MEF2 targets and the aggressiveness of G3 ER$^+$ tumors. The association was further delineated by Kaplan-Meier analysis. High class IIa HDAC expression is associated with reduced survival in patients with ER$^+$ breast cancer.

Although ER positivity is generally considered a favorable prognostic marker, a substantial proportion of patients relapse despite endocrine therapy (31). The genomic grade index (GGI), a signature of 97 genes differentially expressed in breast cancers of low *vs.* high histological grade, has been proposed as a prognostic and predictive factor (32). Interestingly, patients with ER$^+$ tumors and high GGI had worse long-term recurrence-free survival (33). Many genes included in the GGI and also in different prognostic signatures are related to cell cycle and proliferation (34). Likewise, a protein signature of PI3K activation can predict the poor outcome of ER$^+$ breast cancer (35). Interestingly, the expression levels of the two MEF2 target genes KLF2 and NR4A1 inversely correlate with genes that mark cell proliferation in ER$^+$ tumors (Supplemental Table S4). Hence, it will be important to investigate whether connections between class IIa HDACs and

signaling pathways involved in the aggressiveness of ER^+ tumor exist.

How might repression of MEF2 target genes by class IIa HDACs enable more aggressive ER^+ breast cancer and lead to worse clinical outcomes? Experiments in MCF7 cells suggest that class IIa HDACs could affect cell survival. The prosurvival role of class IIa HDACs can be exemplified by the repressive influence on Nur77, a MEF2 target gene controlling apoptosis in certain conditions (29). Certainly, despite the finding, that Nur77 can assume apoptotic functions in MCF7, involvement of other MEF2 targets is highly predictable. Nur77/NR4A1 belongs to the family of orphan nuclear receptors (36). Nur77 can modulate apoptosis through both transcription-dependent and independent activities (37–39). Translocation of Nur77 into mitochondria can convert Bcl-2 into a proapoptotic factor (39), whereas in the nucleus, it can drive the expression of proapoptotic genes (37, 38). Interestingly, the prosurvival role of class IIa HDACs could have a therapeutic perspective. A class IIa HDAC inhibitor (30) elicited an antiproliferative response and apoptosis only in MCF7 and ZR-75-1 ER^+ cells. This response was coupled to the up-regulation of several MEF2 target genes, with Nur77 the more reactive.

In contrast to KLF2, expression of Nur77 was dramatically reduced in MDA-MB-231 cells. This result is not surprising, since MEF2 family members depend on the recruitment of, and cooperation with, other transcription factors to promote transcription of their target gene. In addition, MEF2 target genes (including KLF2 and Nurr77) can be regulated by factors alternative to MEF2, and MEF2 activity can be influenced by mechanisms in addition or alternative to class IIa HDAC binding (40). In fact, MDA-MB-231 cells are also less capable of augmenting Nur77 levels when class IIa HDACs are perturbed. These cells are largely emancipated from class IIa HDACs for the repression of this MEF2 target (41).

Unlike Nur77, in the triple-negative cells and in ER^- tumors, several MEF2 target genes are expressed at higher levels compared to ER^+ tumors. At the moment, the reason for this different behavior is unclear. In basal cancer cell lines, we observed that different alterations in class IIa HDACs could in principle promote the up-regulation of these target genes: point mutations (HCC1937), deficit in nuclear import (MDA-MB-468), reduced deacetylase activity associated with class IIa HDACs (MDA-MB-231). It is also possible that the activation of alterative signaling pathways renders superfluous the MEF2-HDAC axis. In summary, this first work on the MEF2-HDAC axis in breast cancer is clearly exploratory. Nonetheless, data presented here suggest a role of this axis in modulating outcomes in ER^+ breast cancer. FJ

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