UNIVERSITY OF UDINE

DOCTORATE COURSE IN
BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES
CICLE XIV

RESEARCH DOCTORATE THESIS

ALTERATIONS OF THE CLASS IIa HDACs/MEF2 AXIS IN BREAST CANCER CELLS

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ACADEMIC YEAR
2011/2012

Summary

Introduction

Appropriate patterns of gene expression are required for the establishment of specific differentiation programs and for the correct functioning of all cell types. It means that any given cell needs to activate or repress several genes in the right temporal and spatial way. For example, the activation of specific gene programs during development permits the engagement of the differentiation process leading to the generation of cells with highly specialized functions. Moreover, this specific gene expression signature has to be maintained even when inductive signals are terminated. Equally important every cell has also to adapt to any stressful alteration coming from the environment and modulate transcription accordingly. Epigenetic mechanisms, defined as changes in gene expression without changes in the DNA sequence, are ideal for such specific and strict control of transcription (1). Since the epigenetic machinery is involved in so crucial verdicts it is not surprising that in complex diseases like cancer, epigenetic alterations are found to collaborate with gene mutations for tumor growth and development. Importantly, while genetic lesions are permanent, epigenetic aberration can potentially be reversed. Hence every component of the epigenetic machinery could be exploited as a target to be employed in therapy.

In mammalian cells the proper transcriptional process and the specific chromatin state is reached by the work of all epigenetic machineries which exploit different mechanisms like: DNA methylation, nucleosome remodeling and several modification of the amino-terminal tail of histones. One of these modifications, acetylation, can promote gene expression in different ways. Acetylation can neutralize histone positive charges thereby promoting a relaxed chromatin conformation (2) or it can be used as a binding site for a plethora of protein activators, which usually carry a bromodomain, necessary for the interaction (3).

Acetylation is a dynamic/reversible process that allows cells to respond to different extracellular cues. In fact it is controlled by two different groups of enzymes: Histone Acetyl Transferases (HATs) and Histone Deacetylases (HDACs), whose activity serves to regulate gene expression during developmental and disease states. Importantly inhibition of HDACs has emerged as potential strategy to reverse epigenetic abnormalities observed in cancer, with promising results in preclinical models.

Class IIa HDACs are a specific group of Histone Deacetylases

The human genome encodes for eighteen different HDACs, which are grouped in four classes. Different charcateritics regarding structural features, catalytic activity, subcellular distribution and interacting partners allow the juxtaposition of the different HDACs in four different groups (4).

- Class I enzymes embraces HDAC1, -2 , -3 and -8; all the members present high homology with yeast enzyme Rpd3.
- Class II enzymes includes HDAC4, -5, -6, -7, -9 and -10; are structurally related to Hda1 yeast deacetylase.
- Class III enzymes is composed by Sirt1, 2, 3, 4, 5 ,6 and 7.
- Class IV is constituted only HDAC11. This protein shares homology with both Class I and Class II.

In particular Class II can be additionally subdivided in Class IIa which includes HDAC4, 5, 7, 9 and Class IIb represented by HDAC6 and 10.

Catalytic activity of Class IIa HDACs

Class IIa HDACs structure presents a peculiar organization. Contrary to Class I HDACs, which show a relative compact structure, Class IIa enzymes are characterized by a bipartite organization where the catalytic domain is located at the C-terminus. Despite sequence differences, Class I and Class IIa maintain a similar folding of the active site and of substrate binding residues. While the contribution of the deacetylating activity is fundamental for the repressive ability Class I members (5) this is not always necessary for Class IIa. In fact MITR, a splice variant of HDAC9 which lacks the C terminal domain, is still able to act negatively on transcription (6,7).

Meticulous inspection of Class IIa HDACs deacetylase domain identified the presence of an aminoacid change in the catalytic site (8). Specifically an histidine substitutes a critical tyrosine, which is present in Class I and not-vertebrate Class IIa in the active site. The histidine restrains the formation of a transition state stabilization during the course of the deacetylating reaction. From a structural point of view the H residue points its lateral chain towards the solvent and not towards the substrate/inhibitor explaining because all Class IIa HDACs manifest a very low ability to deaceylate acetyl-lysine. Moreover, the restoration of the catalytic activity after introduction of a point-mutation which changes the histidine with tyrosine does not increase the Class IIa HDACs repressive ability, supporting the model in which the removal of the acetyl groups is not the principal way through these enzymes act to affect transcription (8). The C-terminal domain does not exert directly a deacetylating activity nevertheless in required for the overall process. Indeed this

region is required for the establishment of a high molecular weight complex recruiting HDAC3 and the co-repressors SMRT or NCoR. This heavy molecular weight complex manifests deacetylase activity (9). Specifically Class IIa HDACs bind with the C-terminal domain the SMRT/NCoR co-repressors which are usually associated with HDAC3. Abrogation of HDAC3 recruitment in this complex abolishes the deacetylase activity, thus suggesting that the catalytic function associated with Class IIa HDACs depends on the recruitment of HDAC3.

Structural analysis of the C-terminal domain of HDAC4 revealed the existence of an additional difference between Class I and Class IIa HDACs that is based on the presence of a zinc-binding domain (10). In particular, a zinc ion holds together two protein segments, of 17 and 35 aminoacids respectively, that depart from the core molecule interacting with four different residues (His 665, Cys 667, His 678, Cys 751) conserved in all the Class IIa HDACs. Flexibility is an important feature of this domain. Two of the coordination bonds can change, involving two additional residues (Cys 669 and His 672 replaces His 665 and His 678, respectively). This peculiarity is important for the function of these repressors since the substitution of Cys 669 and His 675 with alanine fixes not only the zinc domain, but also the catalytic site. In fact the conformation of the active site of HDAC4 is similar to the one of Class I HDACs, but in the presence of a substrate/inhibitor this region becomes wider.

The conformational shift promotes the binding of HDAC4 to NCoR-HDAC3. For this reason some HDAC inhibitors could promote the dissociation of Class IIa HDACs form their catalytic partners. Conversely compounds that, by targeting the active site, promote the wider conformation could enhance the formation of a heavy, catalytically competent, repressive complex. This could be the case of the inhibitor MC1568, which promotes the interaction of HDAC4 with NCoR/SMRT-HDAC3 (11). Unluckily a strong effect of this compound has been observed only the context of muscle cells, where Class IIa HDACs play a dominant role whereas in other cell types, like breast cancer cells, its action seems quite limited (11,12).

Regulation of Class IIa HDACs

Cells use different strategies to impinge on Class IIa HDACs functions. Transcriptional and post-translation mechanisms have been described to control Class IIa activities under different conditions. An exquisite network of signals can rely on these repressors to modulate the genetic program of a cell.

Regulation

HDAC4 and HDAC7 proximal promoters present a region characterized by high GC content (13,14). Analysis of these sequences identified several binding sites for Sp transcription factors, which associate with these regions and promote Hdac4 and Hdac7 transcription. An exception of this mechanism of regulation is exemplified in denervated muscles. Here Hdac4 levels rise but this effect is not reverted upon mithramycin treatment, a drug able to bind GC rich sequences and to cause a steric hindrance, suggesting that Sp transcription factors are dispensable in this condition (15). In fact in denervated muscles myogenin is required to promote hdac4 transcription possibly trough E box elements found in the promoter of this transcriptional repressor. While Sp transcription factors seem to mediate a constitutive signal, common to several tissues/districts, other mechanisms can additional balance the expression of these regulators. During myoblast differentiation the activation of MEF2 transcription factors promotes the rise of HDAC9, one of its specific regulators, trough the presence of a Mef2 binding site in the promoter (16). This effect is required to guarantee a fine-tuning of gene expression program during differentiation. Moreover the knock-down of a specific Class IIa HDAC protein results in the up-regulation of the remaining members (17).

Post Transcriptional control- Regulation of translation

Another strategy to regulate Class IIa HDACs levels involves post-transcriptional and translational control. Several microRNAs have been recognized to affect the expression of these repressors. Their effect is particularly important during cell differentiation where different players act to timely promote the right gene expression pattern and where miRNAs add robustness to gene regulatory networks. The first report identifying miRNAs targeting Class IIa HDACs was in a model of muscle differentiation. In this context miR-1 was described to selectively down-regulate HDAC4 levels by binding its 3'UTR where different miR-1 binding sites were found (18). The effect was explained as a block in translation, since HDAC4 mRNA levels were not affected by miR-1. After this first observation different reports described the repressive role of miR-1 on HDAC4 also in other cellular context (19,20). MiR-1 is a member of the so called "myoMir" a group of small RNA considered master regulators of the muscle differentiation program. In the myoMir family other miRNAs have been described to alter HDAC4 levels, they are miR-206 and miR-29 (21). Different signaling pathways regulate the expression of these myoMirs. While the levels of miR-1 are sensitive to mTORC inhibition (22), miR-206 and miR-29 are affected by TGF-beta activation and correspondingly both treatments up-regulate HDAC4 in muscle cells (21). Mir-

206 expression is also enhanced during denervation where is up-regulated by MyoD and buffers HDAC4 increase, in order to favor a possible re-innervation (23). Other microRNAs reported to affect HDAC4 levels are miR-140 and miR-365 that are expressed in the cartilaginous tissue (24,25) where they could regulate the onset of endochondral ossification. While the quoted miRNAs preferentially bind the 3'UTR of HDAC4, miR-2861, is able to affect Class IIa HDACs, and specifically HDAC5 levels, by directly targeting the coding region and again blocking the translation (26).

Post Transcriptional control - Stability Control

To add further complexity to the mechanisms of regulation of Class IIa HDACs function also the stability of these proteins is under the control of extracellular signals. The degradation offers additional advantages to the fine tuned regulation of Class IIa HDACs. First it can allow a sustained activation of transcription factors dependent gene programs when specific signals are provided. This fact has been observed in vivo during myofibers switch from fast to slow twitch. In that context Class IIa HDACs degradation via the ubiquitin proteasome system (UPS) is required to permit the myofibers switch, under the control of the transcription factors MEF2s (27). Moreover also TGF-beta stimulation of breast mammary epithelial cells elicits the UPS-dependent down-regulation of Class IIa HDACs (28). Second degradation is a mechanism to control the levels of Class IIa HDACs when they are not longer required or when potentially harmful for the cell, under specific conditions. In fact, upon growth factors deprivation, in untransformed cells HDAC4 is destroyed by the UPS while this modulation is lost in cancer cells (29). The degradation is dependent on the presence of a phosphodegron, a protein sequence that requires the phosphorylation on Ser/Thr to be recognized as signal for the action of the UPS. In particular this phoshodegron is target of the kinase GSK3-beta, which controls stability of several different protein as Jun (30). The HDAC4 mutant S298D, mimicking the presence of a phosphate group transferred by this kinase, is unstable and highly poly-ubiquitinated.

Quite interestingly, two different reports have observed that the degradation of Class IIa HDACs occurs in the nucleus in both muscle fiber switch and serum deprivation (27,29). This localized destruction is probably required to ensure the correct gene activation impacting on the active repressive pool of these enzymes while the sequestered cytoplasmic fraction is preserved in order to control rapid changes in the signal/environment. Unfortunately all papers lack the identification of the E3 ligase enzyme to draw the complete picture. However Ishicawa et al. tested several E3 ubiquitin ligases that are up-regulated after TGF-beta stimulation like Smurf1 and 2, Nedd4,

Arkadia, WWP1 and 2 but none of the tested proteins were implicated in the regulation of Class IIa HDACs stability (28). In the case of the GSK3-beta dependent degradation it is possible to speculate that the destruction involves the SCF family of ubiquitin ligases, since they usually require the presence of a phosphodegron signal.

Subcellular compartimentalization control

Nuclear residency of Class IIa HDACs is necessary to repress transcription. In the nucleus these repressors associate with different binding partners to modulate gene expression. Initial observations, using leptomycin-b, an inhibitor of nuclear export, pointed out the ability of Class IIa to shuttle continuously between the nuclear and the cytoplasmatic compartments (31). The regulation of their subcellular localization is a useful strategy to control their impact on chromatin: to increase their repressive ability it is required the nuclear accumulation, whereas nuclear export is favored when transcription needs to be switched on. The movement between the two subcellular compartments is afforded by a nuclear localization (NLS) and a nuclear export signal (NES) detained by all Class IIa HDACs. The NLS sequence is localized in the amino-terminal domain and is composed by a tripartite arginine-lysine motif (32). Conversely the NES is located at the C-terminal part in the catalytic domain and is represented by an hydrophobic motif able to bind the CRM-1 transporter, which mediates the nuclear exit (32,33). Interestingly the splice variant of HDAC9, MITR, lacks the NES sequence and cannot be exported in the cytoplasm (7). To hold in check Class IIa HDACs the cell exploit conserved serine residues located preferentially in the amino-terminal domain (3 Ser residues for HDAC4, 5, 9 and 4 on HDAC7). Once these residues are phosphorylated, by the action of different protein kinases, they act as docking sites for the binding of 14-3-3 proteins (34). As a consequence the mutation of these Ser residues in Ala, which abolishes the potential phosphorylation, releases Class IIa HDACs from 14-3-3 proteins control and generate enzymes mostly nuclear that exhibit an increased repressive potential (35). The association with 14-3-3 adaptors can exert diverse effects, which contribute to change the subcellular localization. First it can mask the NLS sequence to importin-alpha binding, therefore blocking the import process (34). Second it could promote a conformational change, which fosters the exposition of the NES sequence thereby inducing nuclear export (33). Besides export to cytoplasm Class IIa HDACs are subject to additional levels of regulation. An HDAC5 mutant carrying an inactivated NES cannot exit the nuclear compartment but is unable to impact on transcription, thus suggesting that the sequestration of these enzymes away from their transcriptional partners, probably due to the effect of 14-3-3 proteins, limit their repressive ability (32,36). Furthermore FRAP experiments

provided additional evidences to a nuclear control of Class IIa HDACs function. The forced accumulation of HDAC4 by ratjadone treatment or the mutant carrying a defective NES behave in a similar manner and bind chromatin in a rapid and transient way (37). Conversely the mutant that cannot bind to 14-3-3 chaperones (Triple Mutant, TM) is characterized by a low recovery after bleaching, suggesting a longer binding to chromatin. In this light, post-translational modifications such as phosphorylation or caspase processing can affect not only the nuclear residency but also the binding to chromatin and therefore the repressive influence. Finally also the association with NCoR/SMRT-HDAC3 complex could modulate the residence in the nucleus. In fact this binding overlaps to the NES sequence and can be masked (10). Moreover the formation of the complex could also support the positioning onto chromatin to strengthen the repressive ability, as observed in FRAP experiments (37).

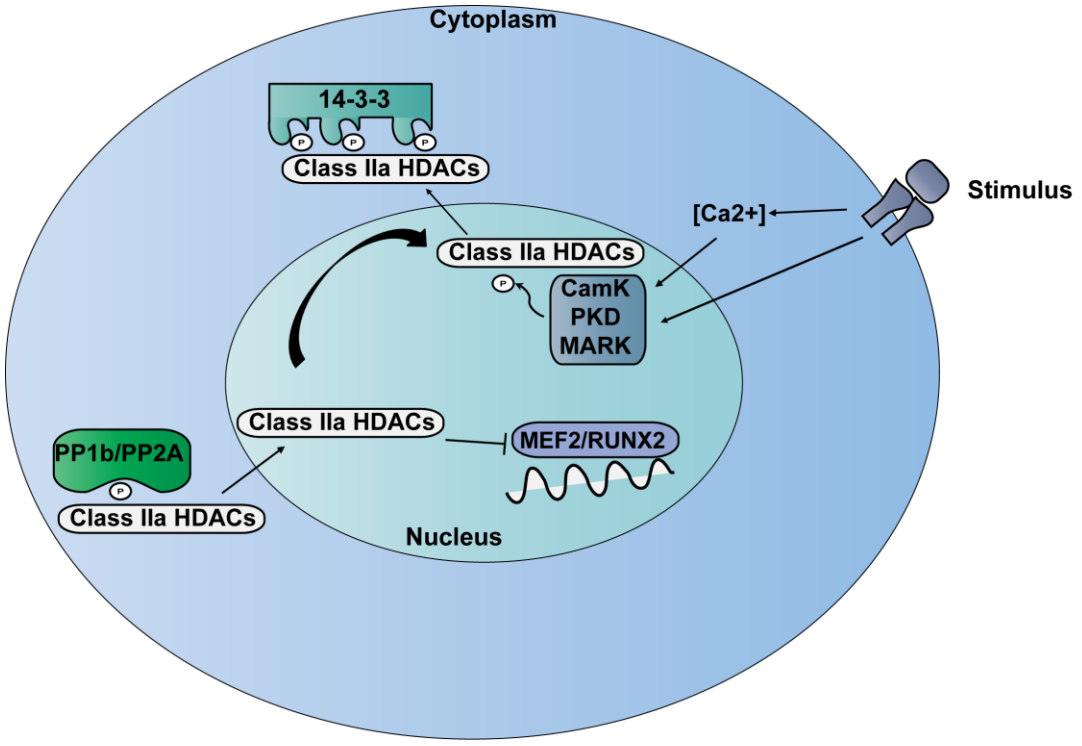
A wide array of signals is able to affect Class IIa HDACs localization. Each of these stimuli is able to activate specific protein kinases, which in turn phosphorylate these deacetylases. Historically the first signal described to modulate Class IIa HDACs nuclear export was calcium release through the action of the Calcium-Calmodulin dependent Kinases (CaMK) (36,38). In particular in a model of muscle cell differentiation the activation of CamKI promotes cytoplasmic accumulation of Class IIa HDACs favoring nuclear export (38). Not only CaMKI but also CaMKIV exerts its effect over these deacetylases (39). While CaMKI and IV affect all Class IIa HDACs, CamKII seems to manifest a degree of specificity since it interacts only with HDAC4 (40). HDAC4 then, thanks to an hetero-oligomerization, is able to integrate the signal coming from this kinase and to extend it on HDAC5 and on MITR and promoting also their nuclear export (40). This mechanism relies on the ability of HDAC4 to interact with HDAC5 and MITR through a coil-coiled region in the amino-terminal domain, conserved among these repressors but not present on HDAC7.
Initial observation also stated that the inhibition of CaMK by the use of the inhibitor KN93 was not enough to block Class IIa HDACs phosphorylation, thus suggesting that additional kinases are involved in the regulation of these deacetylases (41). An additional kinase activated by calcium release is PKD, which is also implicated in the regulation of these repressors (42). Moreover activation of G protein coupled receptors in the myocardium regulate also the kinase (GRK5) which again phosphorylates Class IIa HDACs (43) and as MirK counteracts their repressive ability (44).
Beyond the classical activation of CaMK and PKD, calcium also modulates Class IIa HDACs through an indirect effect. It promotes CREB dependent gene expression which up-regulates Salt Inducible Kinase 1 (SIK1), another Class IIa HDACs kinase (45).

Class IIa HDACs exhibit a nuclear exclusion that can be independent from calcium signaling. Also the metabolic state of the cell influences Class IIa HDACs functions. Upon energy depletion, and the concomitant increase in AMP concentration, AMP-Kinase (AMPK) stimulates the 14-3-3 dependent nuclear export of these deacetylases (17,46). All these enzymes are activated in specific conditions modulating Class IIa HDACs in response to specific external stimuli. However these deacetylases are also controlled at a steady state level by constitutively active kinases belonging to the Microtubule Affinity Regulated Kinases (MARKs). In particular two members, MARK2 and 3, are able to promote the association of the deacetylases with the 14-3-3 proteins (47). Interestingly MARKs, PKD, and CaMK are all members of the CaMK superfamily of enzymes (48). Furthermore SIK1, MARKs and AMPK belong to the AMPK family of enzymes and are all under the control of the master regulator LKB1 (49) implicated in the regulation of both cell polarity and metabolism. Several kinases are implicated in the control of Class IIa HDACs function suggesting that their regulation requires the right spatiotemporal signaling in order to permit the correct gene expression. Moreover redundancy in the activation of these enzymes, like in the case of PKD and CaMK, suggest that more than one pathway is necessary to ensure gene induction in a quick and adequate manner. Moreover redundancy guarantees that the second arm can buffer alterations in the first arm. Signals that drive Class IIa HDACs out of the nucleus are well described, however the link between these signals and other pathways are still not carefully depicted. It is interesting to note that in a screening for additional regulators of these repressors several new players have been identified like ERBB2, PIAS4, SENP2 (50). Unfortunately none of these have been characterized in their detail mode of action.

Beyond the classical model requiring phosphorylation, Class IIa HDACs localization can also be affected by an independent mechanism. In fact the regulation of the intrinsic flexibility of the C-terminal domain of Class IIa HDACs is involved in the control of nuclear localization. Two Cys residues, Cys 667 and Cys 669, controlling the flexibility of the C terminal domain, play a major role in this regulation. In the reduced state, the catalytic domain can interact and mask the NES. Conversely the generation of thiol groups promotes the nuclear export even when Ser/Ala mutations are introduced in the 14-3-3 binding sites (51).

Undoubtedly signals that drive Class IIa HDACs out of the nucleus and permit gene expression are very important. Signals that govern the opposite movement: the nuclear import are not less important. Unfortunately they are much less characterized. Initial observation with the use of chemical compounds like calyculin A supported the involvement of protein phosphatases in the

reinstatement of the transcriptional inhibition of the Class IIa HDACs (34). However, it took several years before having a clear description of this mechanism. The first enzymes identified were the Protein Phosphatase 1-beta (PP1b) and the Myosin Targeting subunit 1 (MYPT1) which are constituents of the Myosin phosphatase (52). Further studies contributed to unveil the role of Protein Phosphatase 2 A (PP2A) (53-55). PP2A competes for the binding of phosphorylated residues with 14-3-3 proteins and mainly interacts with the amino-terminal domain of Class IIa HDACs (54). Its action promotes mainly the dephosphorylation of the first serine residue, which usually is enough to promote cytoplasmic retention of Class IIa HDACs (53). Moreover PP2A affects also the phosphorylation of an additional Ser in position 298 of HDAC4. Although this Ser is not a residue target of kinases regulating the nuclear export, it exerts a fundamental role in the control of nuclear localization. In fact HDAC4 S298D mutant is confined in the cytoplasm also in the presence of a block in the nuclear export (54). Treatment of the cells with leptomycin and inhibitors of the UPS rapidly induces the accumulation of the S298D mutant into the nucleus.
This result indicates that HDAC4 is degraded in the nucleus and that PP2A can regulate also HDAC4 stability via Serine 298.
Different stimulations affect Class IIa HDACs nuclear re-localization. While Nitric Oxide (NO) acts only through PP2A (56), Parathyroid related Peptide (PTHrP) and forskolin stimulate also PKA (55). In fact the rise in cAMP levels promotes Class IIa HDACs nuclear accumulation with the exception of HDAC7 that lacks the PKA consensus motif. PKA phosphorylation site lies in the NLS and is interposed between the first two serines promoting the export (57). It is still debated how this phosphorylation can influence nuclear import. It could affect the 14-3-3 binding, probably causing a steric hindrance or a shift in the structure of Class IIa HDACs. In this way PKA could favor PP2A binding and de-phosphorylation.
Also MEF2 transcription factors are able to induce the nuclear accumulation of Class IIa HDACs (58). In this case MEF2s provide a NLS in trans for the deacetylases and rescue, at least partially, the import even for mutants lacking the nuclear localization sequence (54).



Class IIa HDACs regulation of cellular localization inside the cell
Extracellular stimuli that are able to activate CaMK, PDK and MARK kinases trigger the phosphorylation of class IIa HDACs and their association with 14-3-3 proteins, thereby promoting nuclear export. Conversely, the removal of the phosphate groups catalysed by PP1b or PP2A stimulates Class IIa HDACs nuclear accumulation and repression of MEF2-dependent gene expression.

d-Additional post-transcriptional modifications

Additional post-translational modifications can impact on Class IIa HDACs functions. These deacetylases are target for SUMOylation which occurs in the amino-terminal part of the protein (59). However the contribution of this modification to the repressive ability is not yet clear. Class IIa HDACs can also promote the SUMOylation of MEF2s. SUMO is ligated in the transcriptional activation domain and inhibits transcription (60). Initial experiments suggested a possible E3 ligase activity of HDAC4 but this result was not confirmed (60,61). Certainly Class IIa HDACs enhance this post-translational modification, and with increased capacity when they cannot be modified by SUMO (60).

Proteolytic cleavages can also influence Class IIa functions. During the course of apoptosis caspases cleave HDAC4 and HDAC7. HDAC4 proteolysis is under the control of caspase-3 and 2 produces and an amino-terminal fragment of 289 aminoacids that accumulates in the nucleus (62) where it is able to repress Mef2 dependent transcription. Interestingly this form can bind Mef2 with high affinity but is less efficient in modulating gene expression compared to the wild type, probably due to its high motility on chromatin, suggesting that transient and quick interactions, possibly with multiple partners are necessary for activating the apoptotic program (37). HDAC7 is primarily cleaved by caspase-8 which, similarly to HDAC4, generates a small amino terminal fragment (63).

However this fragment is very unstable and target of additional cleavage events. Similarly to HDAC4 the generated form is less efficient in the repression of Mef2 dependent transcription.
Finally, the impact of kinases on Class IIa HDACs is not limited to the control of shuttling. PKA not only promotes nuclear accumulation of Class IIa HDACs but also controls the protease cleavage operated by still unknown serine-protease (64). This cleavage is produced in the cytoplasm and generates a small amino-terminal fragment which conserves the Mef2 binding site but not those for SRF thus creating a form with a different repressive competences .

Principal interactors

Class IIa HDACs need to reside on chromatin in order to exert their repressive function. Since they lack a DNA binding domain, their recruitment to specific promoters is dependent on their ability to interact with different transcription factors. The interaction with a specific partner defines the repressive competence and the localization on target promoters.
Among the wide list of Class IIa HDACs interactors the best characterized are the transcription factors of the MEF2 family (36,65-67).The binding motif, conserved in all of these repressors, is composed of 12 aminoacids found in the amino-terminal domain (32) separated by few residues from the one for the Serum response Factors (SRF) (64). Both MEF2 and SRF belong to the same MADS box transcription factor family and are required for cell differentiation in several contexts. Another partner of Class IIa HDACs is Runx2 whose association not only repress its transactivating ability but also stimulates its degradation (68,69). Furthermore additional transcription factors are target of the repressive functions of Class IIa HDACs like GATA (70), Forkhead (71,72) Ying Yang1 (73,74) or BCL6 (75), although less studied. In addition also hormone receptors are characterized interactors of these deacetylases, as in the case for the estrogen and the androgen receptors (76-78).
The ability to interact and form multiprotein complexes is of great importance for the establishment of repressive marks on chromatin. Class IIa HDACs not only recruit HDAC3, which contribute to the deacetylating activity on chromatin, but can also interact with additional epigenetic regulators as the H3K9 methyltransferase SUVH1, HP1 (79) or the corepressors BCOR-L1 (80) and CtBP (81).
Interestingly most of the described interactions involve the amino-terminal domain of the deacetylases. A distinct feature of Class IIa HDACs is the presence of a glutamine rich region in their amino terminal part (82). This domain folds forming alpha helix which can give origin to tetramers. Moreover it has been proposed that glutamine rich region could modulate transient protein-protein interactions thus justifying the long list of Class IIa HDACs interactors.

Biological Functions:

1-regulators of development

As most epigenetic regulators, also Class IIa HDACs play a crucial role during development. Most of their biological effects originate by the modulation of genes under the control of their molecular partners. A great contribution in understanding the role of these epigenetic regulators came form the use of KO mice which pointed out a specific need of Class IIa HDACs during development. It is not surprising that most of their documented biological functions rely on the MEF2 regulation since these transcription factors are their best described interactors so far.

The ossification program

HDAC4 expression is widespread in different cell context however its loss is specifically associated with an alteration of the endochondral ossification program (68). As a result HDAC4 null mice die during the perinatal period manifesting several skeletal abnormalities due to premature ossification, which causes problems to the skull, the breathing and the motility. This process is related to a precocious chondrocyte hypertrophy, which is followed by chondrocyte apoptosis. Initially the HDAC4 null phenotype was explained by the altered regulation of Runx2, a central transcription factor that regulates endochondral bone growth. This model has been refined by recent observations, discovering the unsuspected, but necessary role of Mef2 in this developmental process (65). Interestingly hemizygous deletion of MEF2C rescued the phenotypic abnormalities seen in HDAC4 null mice demonstrating the need of a balance between HDAC4 and MEF2C for a correct ossification program.

Endothelial cells and the vascular system

During mouse embryogenesis HDAC7 accomplish an irreplaceable role in endothelial cells. In fact HDAC7 null animals die in uterus because of an alteration in cell-cell adhesion, which affects the integrity of the forming vascular system, leading to dilatations and hemorrhages (67). The phenotype is, at least in part, explained by the over activation of Mef2 factors that causes the up-regulation of matrix metalloprotease 10 (MMP10). Moreover siRNA mediated knock down of HDAC7 in HUVEC cells dramatically affected the generation of capillary structures in vitro confirming the essential role of this enzyme in the regulation of the angiogenetic process (83). This effect is coupled with morphological alteration observed at the actin cytoskeleton. In general all Class IIa HDACs are exported from the nucleus after VEGF stimulation, which triggers the activation of PKD (84). The export is required for the expression of the gene program promoted by

the growth factor. Conversely, HDAC5 overexpression markedly decreases capillary tube formation of endothelial cells in Matrigel he negative effect of HDAC5 over angiogenesis seems not to depend only on Mef2 repression since a mutant unable to interact with these transcription factors still blocks the sprout length (85). Microarray analysis demonstrated the regulation operated by this repressor over the angiogenetic factors FGF2 and Slit2. In addition Class IIa HDACs regulate also gene expression programs of endothelial cells related to changes in blood flow. Shear stress activates HDACs nuclear exit and promotes anti inflammatory responses, observed with the induction of KLF2 (86). Conversely disturbed flow, termed orbital, decreases the phosphorylation-dependent nuclear export and enhances their repressive ability, as evidenced on Mef2 target genes (87).

Heart development and remodeling

Heart formation is initially based on the establishment of cardiomyoblasts from mesoderm cells thanks to the action of transcription factors of the Mef2, Nkx2-5 and GATA families. These TFs are required for the expression of cardiac muscle-specific genes. Overexpression of HDAC4 or its liberation from the shuttling control, block the differentiation of P19 cells in cardiomyoblasts (39). Curiously no alteration in heart development has been detected in any of the single Class IIa HDACs KO mice, probably due to the redundant role of the remaining enzymes. Interestingly the contemporary loss of HDAC5 and HDAC9 causes perinatal death due to heart developmental abnormalities. These abnormalities are a consequence of altered growth and maturation of cardiac myocytes but the mechanism responsible is not well defined (66). Class IIa HDACs not only affect heart development but participate also in cardiac remodeling in adult animals. In fact HDAC5 and HDAC9 null animals are prone to manifest age dependent cardiac hypertrophy. Interestingly in this pathological condition calcium levels are altered and this condition activates PKD and CamK kinases, all involved in the regulation of Class IIa HDACs shuttling (42,88,89). While most kinases control Class IIa nuclear export, activation of CamKII seems to affect the nuclear import impeding it, as the overexpression of the cytosolic CamKII mutant is still able to block Class IIa enzyme accumulation in the nucleus (90). In general the cardiac hypertrophy condition is associated with an increased amount of signals that converge to drive these repressors out of the nuclear compartment. For example, stimuli that affect calcium levels also generate ROS production that favors the exposition of the NES sequence of Class IIa HDACs (51). Conversely the expression of nuclear export resistant enzymes reduces the hypertrophic response . Class IIa HDACs protect against pathological heart remodeling, but their knock down could be beneficial after myocardial infarction. This effect is observed only in female mice and is caused possibly by the MEF2-dependent up-

regulation of vascular endothelial growth factor (VEGF) via the estrogen receptor, which promotes neo-angiogenesis (76).

Skeletal muscle development and remodeling

Mef2s and Class IIa HDACs play a central position also during the skeletal muscle development. Overexpression of these deacetylases or the stabilization of HDAC-Mef2 complex block Mef2 dependent differentiation of myoblasts into myocites (11,91). Conversely exposure of myoblasts to differentiating conditions causes a re-localization of Class IIa HDACs in the cytoplasm and exogenous CaMKs enhance this process (92). It is interesting to note that during the course of differentiation Mef2 is able to drive HDAC9 expression, which suggests the need of precise Class IIa titration to ensure a correct differentiation (16). However, no alteration has been observed in skeletal muscle development in anyone of the HDAC null mice, suggesting the existence of redundant action among these enzymes in the muscle tissue. In addition to the nuclear/cytoplamsic shuttling other strategies are used for the modulation of Class IIa HDACs function in skeletal muscle differentiation. MyoD can stimulate the expression of PC4, a factor that can interact and sequester these deacetylases away from Mef2 (93).

Class IIa HDACs participate in the regulation of skeletal muscles also in the adult age. In particular HDAC4 is up-regulated after immobilization, condition that comes along with a decrease in neuromuscular activity (94). This effect is also found after denervation, a state observed in some neurodegenerative diseases like ALS or NMD (94). Curiously, in mice after denervation, HDAC4 shift subcellular localization being principally nuclear and controls neuromuscular gene expression (15,94). In particular it represses HDAC9 and Dach2 expression and in this way liberates Myogenin from its controllers. Myogenin can stimulate the expression of atrogin and murf1, two E3 ligases involved in the atrophic process (95). Interestingly mice null for HDAC4 and HDAC5 are partially protected from denervation induced muscle mass loss.

Lymphocytes

Class IIa HDACs affects also the development and the functions of the immune system,. In order to avoid potential problem of autoimmunity, T cells that displays a strong interaction with MHC complex coupled with a self-peptide are eliminated. This negative selection takes part in the thymus and involves T cells that express both CD4 and CD8 markers (96). HDAC7 is the most expressed Class IIa deacetylases in double positive thymocytes and regulates the apoptotic susceptibility of these cells. By negatively regulating Mef2, it represses the expression of the pro-apoptotic protein

Nur77 (96). The engagement of the T cell receptor determines the export of HDAC7 and the consequent de-repression of Nur77, which can kill self-reacting lymphocytes (97). Moreover HDAC7, beyond its control of the survival in developing T cells, can influence also the differentiation favoring the generation of CD8 positive cells. The regulation of this protein is also important in cytotoxic T lymphocytes where the expression of a nuclear export defective mutant restrains proliferation, as mediated by IL2 (98). HDAC7 is not the only Class IIa HDAC involved in the regulation of T cells. HDAC9 plays a central role in the control of T regulatory cells (Treg). HDAC9 KO mice present higher number of Treg and increased immune suppressive functions. This effect is due to the unrestricted activity of Foxp3, an important transcription factor for development and function of these regulatory cells (72). Finally also in B cells the engagement of the B cell receptor promotes the activation of PKD, which drives Class IIa HDACs out of the nucleus (99) although their biological role in this context is not yet depicted.

Adipocytes

Also the differentiation of adipocytes is under the control of Class IIa HDACs and specifically of HDAC9 and its splice variant MITR. In mesenchymal stem cells (MSC) MITR expression enhances the generation of osteoblasts and represses the formation of adipocytes through the repression of the PPAR-gamma transcriptional program (100). However, MITR levels are controlled by another epigenetic regulator, EZH2, which methylates it and in this way favors the differentiation of MSC in adipocytes. Moreover also HDAC9 controls the transition of pre-adipocytes to adipocytes and its loss enhances this process quantified with the accumulation of lipid droplets (101). Its expression is negatively modulated during the differentiation and is transcription dependent. The blockade of adipocytes differentiation by HDAC9 is dependent on its repressive role on the USF-1, key transcription factor of this process.

2-Regulators of metabolism

Several kinases like LKB1/AMPK, MARK2 or SIK1 that hold in check Class IIa HDACs are also well known regulators of energy production and metabolic processes (102,103). Hence it was not surprising to discover that Class IIa HDACs govern central aspects of metabolism considering that they can impact on gene expression in different tissues, from muscles to adipocytes.

In the muscle, the degradation of Class IIa HDACs is coupled with the switch from fast twitch myofibers, generally glycolytic, to slow type contraction preferentially oxidative. This effect is

usually observed as result of training and is related to the activation of Mef2 (27). Similar results have been observed in the heart where the overexpression of the constitutive active form of HDAC5 causes dramatic alterations in mitochondria, which become swollen with altered cristae (104) . These changes are coupled with the down-regulation of different enzymes involved in fatty acid oxidation. This fact also promotes the accumulation of lipid droplets in cardiomyocytes and induces the expression of PPAR-gamma, probably as a compensatory mechanism. Moreover, also the glycogen lysis is affected with the down-regulation of glycogen phosphorylase and there is a limit in the glycolysis with the decrease of hexokinase II. Additional studies noticed the repressive effect of HDAC4 on glycolysis. During denervation HDAC4 is recruited to the promoters of MSE, PKH and GLUT4 and its knockdown increases the amount of piruvate produced (15). The control of GLUT4 expression by Class IIa HDACs is evidenced also in adipocytes where these repressors reduce its levels, by affecting the transactivating ability of Mef2 and Gef 2 TFs (105). In these cells, HDAC9 was described as an important regulator of expansive processes, as it blocks lipogenesis during fasting (106). The repression of USF-1 transcription factor explains the role of HDAC9 in the regulation of lipogenesis, while its recruitment to chromatin is blocked by insulin stimulation, which signals nutrients availability. Fasting is a condition that affects Class IIa HDACs in another district, crucial for body metabolism and homeostasis: the liver. Here these deacetylases act by mediating the role of glucagone, which activates PKA, and stimulate their nuclear entry, where they promote gluconeogenesis (17). Conversely, in the liver the insulin stimulation or the activation of LKB1/AMPK pathway block this process favoring the export of Class IIa HDACs. Their nuclear accumulation is coupled with the induction of gluconeogenetic target genes, like GP6ase and Pepck. ChIP studies highlighted the recruitment of deacetylases in a region containing Foxo binding sites. Interestingly, Foxo DNA binding is negatively affected by acetylation. Class IIa HDACs, via HDAC3 action, liberate Foxo from this modification and promote its recruitment to chromatin in association with these repressors. Since high Foxos activation is characterized for insulin resistance, the modulation of Class IIa HDACs could be of potential interest for metabolic diseases.. In fact, knock-down of these repressors in obese animals or mice treated with high-fat diet (mimicking diabetes type 2) reduced the glucose concentration in the bloodstream.

All these observations suggest that Class IIa HDACs control energy generation in different ways affecting both aerobic and anaerobic systems and affecting also the storage of nutrients in different districts. Finally, the effect of Class IIa HDACs in hypoxic conditions is rather different. In fact Hdac4, blocking the degradation of Hif1- alpha increases glycolytic metabolism (107).

Class IIa HDACs and cancer

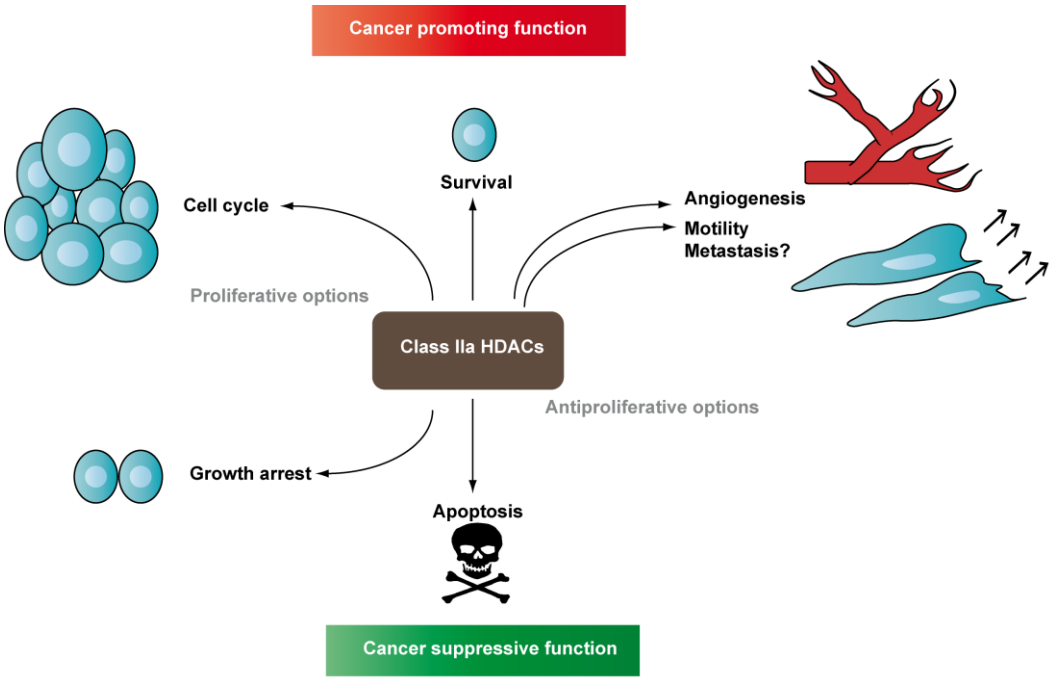
Class IIa Histone Deacetylases roles in neoplasia are not clear depicted. Some reports described them as positive regulators of tumor growth while others evoke an anti-proliferative signaling. One possible explanation of their behavior could stem on the context dependent status of different post-translational modifications that regulate their functions. In addition also the level of activation or the mutational status of their binding partners could vary among different cancer types could influence the contribution of Class IIa HDACs to neoplastic growth. The advent of the new generation sequencing and the development of ChIP assay will contribute to better delineate their contribution to tumorigenesis.

Mutations

With the beginning of cancer genome sequencing projects a lot of information are available regarding the mutational status of genes in human tumors, including Class IIa HDACs. Initial observations pointed-out significant mutations of HDAC4 in human breast cancer samples (108) . In addition HDAC4 has been found mutated also in melanoma (109). Subsequent analysis made in head and neck squamous cell carcinoma reported different mutations of HDAC7 and HDAC9 (110). However data from The Cancer Genome Atlas highlight several mutations in all Class IIa members in colorectal carcinoma, with HDAC9 the most targeted. Additional alterations are found in HDAC4, HDAC5 and HDAC7 in serous ovarian carcinoma (http://www.cbioportal.org/public-portal/). Interestingly HDAC9 is frequently associated with SNP mutation in lung cancers with mutated EGFR and is significantly amplified in sarcoma samples (111) (http://www.cbioportal.org/public-portal/). The great wealth of information obtained from genome sequencing projects are undoubtedly important, however the contribution of every alteration need to be understood deeply to assess the specific role in the specific context..

Correlation with tumors

Although the involvement of Class IIa HDACs in cancer is not well depicted, some studies reported an association between their expression and specific neoplasias. In general the increased expression is usually correlated with poor prognosis. This is the case of medulloblastoma where HDAC5 and HDAC9 have augmented expression (112). Similarly high HDAC7 and HDAC9 levels are associated with bad prognosis in acute lymphoblastic leukemia (113). High cytoplasmic signals have been described for HDAC7 in pancreatic cancer but its association with survival has not been characterized (114). Instead in colorectal tumors and in glioblastoma HDAC5 and HDAC9 expression levels are lower (115,116).



Class IIa HDACs functions observed in cancer cells
Schematic representation of the different influences exerted by Class IIa on cancer-related cellular functions. Class IIa HDACs can participate in different cancer-related processes. According to the context they could behave as tumor promoter or tumor repressive players. In the chart are summarized data on Class IIa HDACs status in human cancer.

Pro-oncogenic functions

Studies that support Class IIa HDACs as oncogenes demonstrated their involvement in regulation of cancer cell proliferation. Using an insertional mutagenesis screening approach, HDAC7 has been identified as a potential oncogene in hematopoietic tumors (117). HDAC7 is also involved in the regulation of estrogen-mediated proliferation, recruited to the promoter of Reprimo, a repressor of cell cycle (118). Additional evidences support this concept, as for the absolute requirement of HDAC4 in p53 null cells (119). In this context HDAC4 is necessary to promote efficient mitotic segregation of chromosomes and its down-regulation induces G2 arrest and cell death. HDAC4 is enriched in the proliferating zone of the colon crypts and is required for the negative modulation of the Cdk inhibitor p21 (120). Its recruitment on p21 promoter does not involve the p53 binding sites but the Sp1/Sp3 sites and its silencing inhibits cancer cell growth in vitro and in vivo (120,121).
Deregulated expression of this enzyme was observed in hepatocellular carcinoma where its levels are increased because of the lack of two different miRNAs: miR-1 and miR-22 (19,122). Also in this context the down-regulation of HDAC4 reduces the growth rate (122). miR-1 dysfunction and HDAC4 up-regulation have been reported also in lung cancer cells (20). Deregulation of HDAC4 expression can also come from alteration in its regulatory pathway dependent on the proteasome. Growth factors can augment its levels blocking its poly-ubiquitination (29). However, cancer cells have lost this regulation and HDAC4 is maintained even in absence of growth factors. This up-

regulation has also been linked to resistance to chemotherapeutic drugs. In cisplatin resistant ovarian cancer cells the up-regulation of HDAC4 promotes STAT1 nuclear entry and function, abrogating drug sensitivity (123). Following down-regulation of this deacetylase, STAT1 is maintained in the cytoplasm and resistance to therapy is lost.

Another potential harmful consequence of high HDAC4 expression is its ability to rescue HIF1-alpha form degradation (107,124), stimulating in this way an increase in glycolytic metabolism and resistance to paclitaxel induced apoptosis. HIF1-alpha involves also HDAC7 for the down-regulation of cyclin D1, partially explaining the chemoresistant effect observed with hypoxia (125). These observations suggest that the combination of classical chemotherapy with agents that target histone deacetylases could restore drug sensitivity. Furthermore, the survival role promoted by Class IIa HDACs can be observed also in absence of HIF1 alpha. This is the case of cutaneous T cell lymphoma where HDAC7 represses the expression of the pro-apoptotic gene NUR77/NR4A1 (126).

Despite several observations describing the tumorigenic role of Class IIa HDACs, little is known about how classical cancer signaling pathways modulate their functions. Activation of RAS has been reported to promote nuclear localization of HDAC4 but how this impact on transformation is not known (127). Conflicting observations concern the PI3K pathway, proposed as both activator and repressor of HDACs mediated functions (87,128). Class IIa HDACs however could potentially modulate the activation of the PI3K/AKT signaling repressing PTEN. HDAC5 is in fact involved in the regulation of the phosphatase expression in neural stem cells with the co-repressor TLX (129), but also without its contribution in pancreatic cancer cells (130). In the same direction it has been shown that HDAC7 knock down in endothelial cells decreased the amount of phosphorylated Akt, although the mechanism has not been fully elucidated (83).

Oncosuppressive functions

Initial reports on Class IIa HDACs functions depicted these repressors as onco-suppressor genes. HDAC4 was described as a component of the p53 pathway governing cell cycle arrest (131). Further studies have demonstrated additional connections between HDAC4 and p53, but unfortunately, a clear picture is not available at the moment. HDAC4 can be recruited to G2/M promoters upon DNA damage, as the result of a complex formation with NF-Y and p53 (132,133).

On these sites HDAC4 could exert its repressive influence. HDAC4 was also reported to interact with p53BP and being involved in the DNA repair after ionizing radiation (134). Indirectly also HDAC9 can affect the cell cycle via the p53 p21 pathway (135). It associates and promotes

deacetylation of ATCD/TRIM29 (ataxia telangiectasia group D-complementing) and affect ATCD binding to p53. In this way HDAC9 liberates p53 favoring its activity. However Class IIa HDACs can exert an anti-proliferative role independently from p53. In fact overexpression of HDAC5 or HDAC7 block growth in different cell lines (136). Conversely stimulation of VEGF-induced proliferation drives Class IIa HDACs out of the nucleus, effect lost with export resistant mutant of HDAC7 (84). VEGF stimulates not only HDAC7 export but also its degradation (137). This regulation is important to avoid HDAC7 dependent retention of b-catenin in the cytoplasm. The loss of this repressor allows b-catenin entrance in the nucleus and the G1/S transition. The repressive ability of Class IIa HDACs can affect also steroid receptors. In prostate cancer cells HDAC4 promotes the SUMOylation of the androgen receptor, and in this way the activation of the receptor is reduced (78). Finally Class IIa HDACs can also participate in the apoptotic process, as the amino terminal fragment that originates after caspases processing enhances cell death (62).

Materials and Methods

Cell culture, infections an siRNA transfection and cell death

MCF-10A cells were maintained in Ham's F12/DMEM 1:1 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2mM), insulin (0.01mg/ml), hydrocortisone (500ng/ml), epithelial growth factor EGF, (20ng/ml) and cholera toxin (100ng/ml). 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 that were grown in RPMI 1640 supplemented with 10% FBS plus penicillin/streptomycin and L-glutamine. All supplements were from SIGMA. The CRM1 inhibitor, leptomycin-B (LC laboratories), was used at 5ng/ml. AICAR (SIGMA) was used 100 uM. MCF-7 and MDA-MB-231 cells expressing GFP or HDAC4-GFP transgenes were generated by retroviral infection as described previously (29). Stealth RNAi oligos were purchased from Invitrogen (Life technologies) and used for silencing experiments. Cells were transfected 24 h after plating by adding the OptiMem medium (Life technologies) containing Lipofectamine (Life technologies) plus the stealth RNAi oligos. After 48h from transfection cells were collected. Cell death was scored with Trypan blue staining. Briefly cells were detached with trypsin, resuspended in PBS and then incubated with Trypan blue (0,05 %w/V in PBS) for 5 minutes. Then cells were counted

Immunohistochemistry

Tissue samples were fixed in 10% neutral buffered formalin and paraffin embedded. Four-micrometers-thick sections of breast tissue were deparaffinized and rehydrated, subsequently the slides were microwave treated in citrate buffer pH6 (DakoCytomation), for a total of 20 min brought to room temperature and wash in PBS. After neutralization of the endogenous peroxidase with H2O2 for 10 minutes, the sections were first incubated with protein block Novocastra (UK) for 10 min. Sections were incubated with the primary monoclonal antibodies anti-human HDAC4 (dilution 1:100). Incubation time was overnight at 4°C. Normal mouse serum was used instead of primary antibodies as negative control. Staining was performed by streptavidin-Hrp/biotyn detection system (LSAB+System-Hrp, Dako) and it was completed after incubation with DAB (3,3'-Diaminobenzidine, Novocastra,UK) substrate-chromogen including 3% H_2O_2. After counterstaining with hematoxylin (Novocastra,UK), the sections were viewed under a Leica

DM3000 optical microscope (Leica, Germany) and captions were collected using a Leica DFC320 digital camera (Leica).

Immunoblotting, immunoprecipitation and immunofluorescence

Immunoblotting was performed as previously described (62). Antibodies used in this work were anti: HDAC3, HDAC5 and HDAC7 (Cell signaling), HDAC9 (Abcam), pan-HDACs (Genescript), MEF2A (Santa Cruz), MEF2C (Cell signaling), MEF2D and Ran (BD Biosciences), EFGR (Santa Cruz), Bcl-2 (Sigma).

Immunoprecipitations were carried out as described (29). Briefly, cells were collected directly from culture dishes with a rubber scraper into low salt lysis buffer (20mM TrisHCl pH 7.5, 2mM EDTA, 10mM $MgCl_2$, 10mM KCl, Triton-X 100 1%) supplemented with protease inhibitors. Lysates were incubated with antibody against HDAC4. After incubation with protein A beads (GE), washes were performed with lysis buffer. Histone deacetylase assay was carried out using the HDAC fluorogenic assay kit (BIOMOL). HDAC4 immunoprecipitations were resuspended in the HDAC assay buffer (50mM TrisCl pH8, 137mM NaCl, 2,7mM KCl, 1 mM $MgCl_2$) and incubated with Fluor de Lys Green Substrate for 30 minutes at 37°C. TSA was used at 40μM final concentration.

For immunofluorescence cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton-X100 in PBS for 5 min. Next, coverslips were incubated with primary antibodies. Anti-HDAC4, anti-SMAC (62,138), anti-DRP-1 (BD Bioscience) or anti-STAT3 (Santa Cruz), Finally they were washed twice with PBS and incubated with 488-Alexa or 546-Alexa conjugated secondary antibodies (Life Technologies) and TRITC-phalloidin (Sigma). 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). For retrotranscription reaction MMLV reverse transcriptase (Life Technologies) was used following manufacturer protocol, utilizing 1 ug of total RNA for reaction . q-PCR were performed using the Bio-Rad CFX96 (BioRad) and SYBR green technology (Kapa). Data were analyzed with the Delta Delta Ct method using the geometric mean of HPRT and b-ACTIN for normalization. qRT-PCR data with the inhibitor were obtained using the geometric mean of HPRT, b-ACTIN and GAPDH for normalization. All reactions were done in triplicate.

Genomic DNA isolation, DNA sequencing

Genomic DNAs form the different breast cancer cell lines were isolated and purified using Qiagen Blood and Cell culture DNA kit (Qiagen), PCRs were made using primers covering the different exons. All PCR products were sequenced with the kit Big Dye® Terminator Sequencing RR-100 on ABI PRISM™ 310 Genetic Analyzer platform (Applied Biosystem) on both strands.

Chromatography

Cells were collected and then lysed in the following buffer 50mM TrisHCl pH7.5, EDTA 0,5mM, 120mM NaCl, NP40 0,5%. After centrifugation at 12000 rpm for 10 minutes the extracts were loaded on a column packed with Superose 6 (GE). As running buffer 50mM TrisHCl pH7.5, EDTA 0,5mM, 120mM NaCl, NP40 0,1% was used and fractions of 0,5 ml were collected.

Cell cycle analysis

Before measuring cells were detached by trypsin and fixed in 70% ethanol. After some washing cells were resuspended in PBS supplemented with Triton-X 100, 1% and RNAse-A 100 ug/ml and incubated 30 min at 37°C. DNA staining was performed incubating cells with propidium iodide at 50ug/ml for 45 min at RT. Cells were then passed through a flow cytometer equipped with the Cell Quest software by using a 488-nm argon ion laser (FACScan, BD Biosciences). A minimum of 10.000 events per sample was analyzed. Data analysis was performed by MODFIT software.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states. All analysis were performed using the GSEA software available at 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). For mathematical model explanation refer to (139). At least 1000 permutations were performed using "genes_set" permutation type for data obtained from cell lines or phenotype permutation type for data obtained for human tumors. Dataset for human tumors were taken from GEO database http://www.ncbi.nlm.nih.gov/geo/ . For cell lines were used dataset of Mori GSE15026 (140) and Varma GSE32474 (141). For human tumor samples were employed dataset of Desmedt GSE7390 (142) and Pawitan GSE1456 (143).

TCGA Kaplan-Meier Analysis

Class IIa HDACs expression data were retrieved from the cBio Cancer Genomics Portal (http://www.cbioportal.org/public-portal/). Patients were subdivided into two classes. The high expressing HDACs group was defined if one or more Class IIa deacetylases was found up-regulated (Z score >2) in microarray or Rna-seq data with no concomitant down-regulation of the resting members (Z score 2<Z<-2). The second group was specified as specimens with no alteration in Calss IIa HDACs expression (Z score 2<Z<-2). All estrogen receptor positive tumor samples were taken from the PAM50 Luminal gene expression signatures.

Statistical analysis

Two tailed T test was used to assess the significance for cell count, qRT-PCR data and deacetylase assay. * p< or =0,05, ** p<0,01, ***p<0,005.

Kaplan-Meier survival curves were generated with cBio Cancer Genomics Portal . Statistical significance was assessed using the non-parametric Log rank test p values < or = 0.05 were considered significant. GSE analysis statistics was calculated with GSEA software applying the Kolmogorov-Smirnov non-parametric test.

Results

Class IIa HDAC/Mef2 axis expression characterization in breast cancer cells

Since human breast cancer is an heterogeneous disease we selected a panel of breast cancer cells lines, which recapitulate the most common features and alterations found in human mammary neoplasia (Table 1). The expression of Class IIa HDACs, together with Mef2 transcription factors was analyzed in order to discover if, one or more of these regulators, could be associated with specific cancerous traits. Initially we considered a group of defined luminal cell lines, which express the ER (Neve et al.), compared to the triple negative cell model MDA-MB-468. In general we noted that both Mef2 transcription factors and Class IIa HDACs are expressed. Interestingly, two enzymes: HDAC4 and HDAC7 showed a clear increase in the ER negative cell line (Figure 1A). Conversely none of the Mef2 transcription factors demonstrated a specific pattern of expression.

Next we extended the analysis on the Mef2/HDACs axis by comparing the luminal MCF7, the triple negative MDA-MB-231 and the untransformed MCF10a cell lines. Figure 1B shows that HDAC4, HDAC5 and HDAC9 are expressed at a lesser degree in non transformed cells compared to MDA-MB-468, while Mef2A and Mef2D manifest a complementary pattern.

Subsequently we employed a panel of different ER negative cells characterized for the lack or the amplification of ERBB2. HDAC4 manifested a similar pattern of expression in all the ER negative cell lines with the exception of the BRCA1 mutated HCC1937 cells, in which HDAC7 is highly expressed (Figure 1C). Again, Mef2A and Mef2D show complementary levels of expression in certain breast cancer cell lines. Finally, using an antibody generated against a conserved epitope among HDAC4, HDAC5 and HDAC9 we obtained a pattern very similar to the one of HDAC4 in most cell lines suggesting that this deacetylase is predominant over the other enzymes. Only MDA-MB-157 differed indicating that HDAC9 expression is relevant this cell model.

Analysis of HDAC4 sequence in breast cancer cell lines

As HDAC4 can carry somatic mutations in human breast tumors (Sjoblom et al), we decided to investigate its status in cancer cell lines. We analyzed the coding region, spanning exon 2 to exon 27. Homozygous mutations were found in HCC1937, ZR-75-1, SK-BR-3, and the MDA-MB cell lines (Table 1). Nevertheless only in the first we observed a missense mutation, A786T, in exon 18.

CELL LINE	ER STATUS	PR STATUS	ERBB2 AMPLIFICATION STATUS	GENE EXPRESSION SUBTYPE	HDAC4 MUTATION
MCF10a	-	-	-	Basal B	
MCF7	+	+	-	Luminal	
T47D	+	+	-	Luminal	S874S
ZR75-1	+	-	-	Luminal	
SK-BR-3	-	-	+	Luminal	S874S
HCC1937	-	-	-	Basal A	A786T
MDA-MB-468	-	-	-	Basal A	P855P
MDA-MB-157	-	-	-	Basal B	P855P
MDA-MB-231	-	-	-	Basal B	P855P

Table 1 Description of the panel of breast cancer cell lines employed for the study.
Cell lines are described for their features as expression of steroid hormone receptors like estrogen receptor or progesterone receptor, for the amplification of ERBB2 oncogene and for their gene expression profile according to previous observations (144). Mutation on HDAC4 are ported.

Analysis of subcellular localization of HDAC4 in breast cancer cells.

Beyond expression levels another way to regulate Class IIa HDACs is the control of their subcellular distribution. For this reason we carried out an immunofluorescence assay and analyzed the localization of HDAC4 in all the cell lines tested. Furthermore to evaluate the nuclear/cytoplasmic shuttling we treated cells with leptomycin B, an inhibitor of CRM-1 dependent nuclear export. In all cancer cell lines HDAC4 manifested a diffuse nuclear/cytoplasmic (pancellular) staining whereas MCF10a characterized for a dominant nuclear signal (Figure 1D).
The block of the nuclear export induced a rapid nuclear accumulation with the exclusion of MDA-MB-468 cells, thus indicating that the deacetylase is subjected to nuclear/cytoplasmic shuttling (Figure 1D-1E). To investigate the impairment in the nuclear accumulation of HDAC4 in MDA-MB-468 cells we compared the shuttling of STAT3 through the time, to comprehend whether there is a general defect in the import machinery or rather a specific alteration in HDAC4 import. STAT3 is most entirely nuclear just after one hour of leptomycin B treatment, whereas HDAC4 required three hours to accumulate in the nuclear compartment (Figure 1 F). These results suggest a selective impairment of HDAC4 nuclear entry.

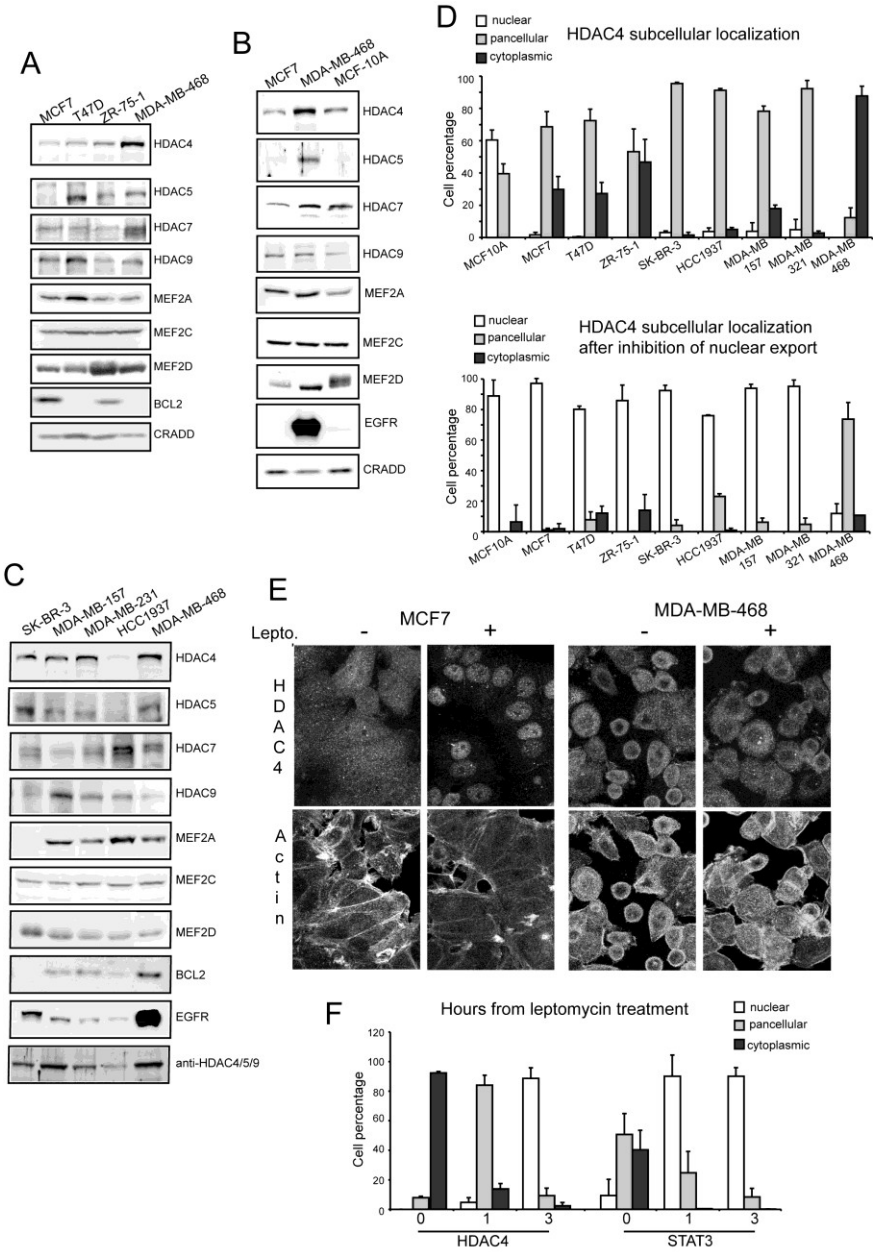
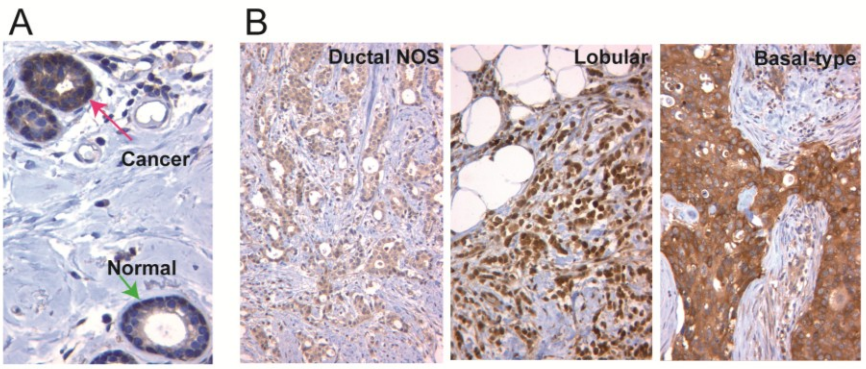


Figure 1. Analysis of HDACs Class IIa expression in breast cancer cell lines.
A) Cellular lysates were generated from the indicated breast cancer cell lines and subjected to immunoblot analysis using the specific antibodies. CRADD was used as loading control.
B) Cellular lysates generated from the non-transformed mammary epithelial cell line MCF-10A and the breast cancer cell lines, luminal type MCF-7 or basal-type MDA-MB-468 were subjected to immunoblot analysis using the specific antibodies. CRADD was used as loading control.
C) Cellular lysates were generated from the indicated breast cancer cell lines and subjected to immunoblot analysis using the specific antibodies.
D) Quantitative analysis of HDAC4 subcellular localization in a panel of breast cancer cell lines and in MCF-10A cells. Immunofluorescences were performed as described in MM to visualize HDAC4. When used, leptomycin B (5ng/ml) was added for 1 h. Approximately 300 cells, from 3 independent experiments, were scored. Data represent arithmetic means $\pm$ SD.
E) Confocal pictures exemplifying the subcellular localization of HDAC4 in breast cancer cell lines. leptomycin B (5ng/ml) was added for 1 h as indicated. Immunofluorescence analysis was performed to visualize HDAC4 subcellular localization. TRITC-phalloidin was used to decorate actin filaments.
F) Quantitative analysis of HDAC4 and STAT3 subcellular localization in MDA-MB-468 cells, after immunoflorescence analysis. Cells were treated with leptomycin B (5ng/ml) for the indicated times. Approximately 300 cells, from 3 independent experiments were scored. Data represent arithmetic means $\pm$ SD.

Class IIa HDACs in human breast tumors

The expression of HDAC4 was also analyzed by immunohistochemistry in 21 human breast cancers. Ducts with signs of malignant transformation show an augmented immunoreactivity for HDAC4 (Figure 2A). However there is no association between HDAC4 levels with a particular cancer subtype (Figure 2B). In Figure 2C are summarized the results of the observation conducted on the selected 21 human breast cancer cases. We evaluated the intensity and the localization of the staining for HDAC4 in an heterogeneous group of samples. No clear correlation was noted between HDAC4 expression and a specific type of tumor or proliferative rate. Moreover, there is no association between HDAC4 levels and the expression of the estrogen and progesterone receptors. Also the subcellular localization shows profound variation across the samples. Interestingly HDAC4 is confined in the cytoplasm in tumors with elevated levels of the ERBB2 oncogene. In general the immunohistochemichal analysis of human cancer samples reflected the heterogeneity found in cancer cell lines. Similar results were obtained after the investigation of public available microarray dataset in GEO and Oncomine for HDAC4 mRNA levels, confirming its variability across samples. Next we interrogated the CBIO Cancer Genome Portal (http://www.cbioportal.org/public-portal/) to use data obtained from The Cancer Genome Atlas (TCGA). We divided human specimens taken from the Cancer Genome Project in two classes to analyze if Class IIa expression is associated with a differential overall survival rate. The first group is characterized for increased amount of at least one Class IIa HDACs (Z score >2), since every member is capable of repressing transcription. The second group conversely does not present alterations in Class IIa members (Z score 2<z<-2).Interestingly we noticed that ER positive tumors with altered expression of Class IIa HDACs displayed a significant decrease in overall survival using either RNA-seq data or Agilent Microarray data (Figure 2D, E) suggesting a selective requirement for these deacetylases.



C

HDAC4 positivity in breast tumors

Patient	Tumor	ER	PgR	ErbB2	KI67*	HDAC4 Intensity*	Nuclear§
900001	Ductal	90%	80%	0	<10	3	20
730002	Ductal	-	-	0	50	2	20
903003	Ductal	nd	nd	1	nd	1	50
812004	Ductal	nd	nd	1	nd	2	100
136005	Ductal	90%	90%	1	<10	1	10
897006	Ductal	90%	90%	0	50	1	50
971007	Ductal	80%	60%	1	<20	2	30
838009	Ductal	nd	nd	3	nd	1	5
267010	Lobular	90%	90%	3	<10	1	0
270011	Lobular	90%	90%	0	<10	3	70
532012	Lobular	40%	40%	1	nd	2	100
271013	Lobular	100%	100%	0	<10	2	100
297014	Lobular	90%	70%	0	5	1	30
297015	Lobular	90%	<10%	0	5	1	50
223016	Lobular	60%	<10%	3	10	1	5
716017	Triple-negative	-	-	0	60	2	40
294018	Triple-negative	-	-	0	nd	2	20
263719	Triple-negative	1%	1%	0	80	3	60
271620	Triple-negative	-	-	0	40	3	30
207121	Triple-negative	nd	nd	nd	nd	3	10
136022	Triple-negative	-	-	1	50	1	0

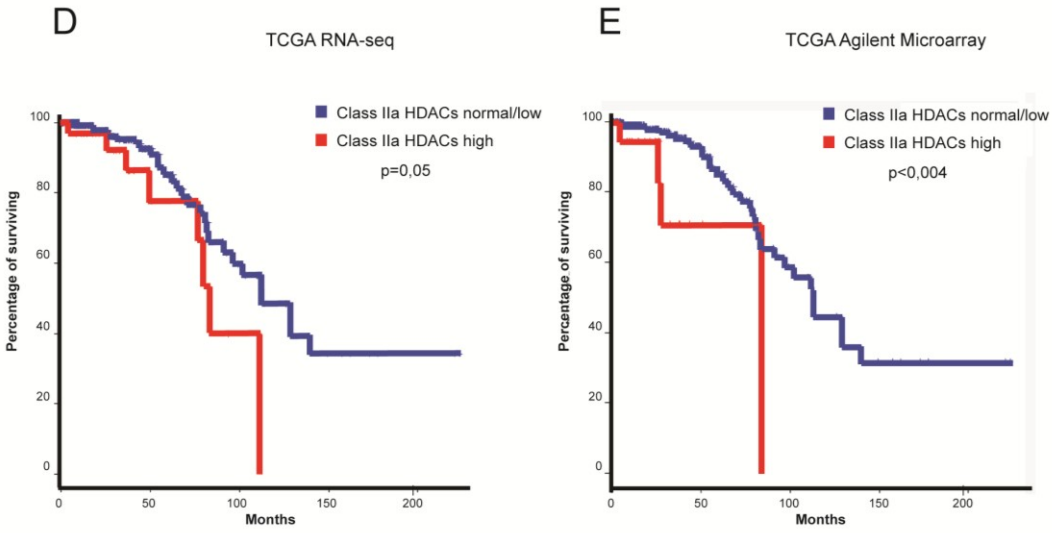


Figure 2 Class IIa HDACs analysis in human breast cancer specimens

A) HDAC4 expression was barely detectable in normal ductal epithelium, while increasing in ducts with signs of malignant transformation.

B) Representative images showing HDAC4 levels in different breast cancers.

C) Summary of the Immunohistochemical analysis. HDAC4 expression in invasive breast cancer varied among different histotypes and showed a combined cytoplasmic and/or nuclear pattern.

D), E) Kaplan Meyer analysis of human breast cancer specimens taken from TGCA database analyzed with RNA-seq or Agilent microarray platform for the expression of Class IIa HDACs. The high expressing HDACs group was defined if one or more Class IIa deacetylases was found up-regulated (Z score >2). The second group was specified as specimens with no alteration in Calss IIa HDACs expression (Z score 2<Z<-2). All estrogen receptor positive tumor samples were taken from the PAM50 Luminal gene expression signatures.

Class IIa HDACs influence on MEF2 dependent transcription in breast cancer cells

Because multiple alterations (nuclear/cytoplasmic shuttling, expression levels, point mutations) could potentially affect the MEF2-HDACs axis and HDAC4 in particular, definition of a 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 function. According to this hypothesis, we decided to use a different approach to unveil the contribution of HDAC4 and Class IIa HDACs to breast cancer. We decided to use the HDAC4-repressive activity as a tool to unveil its contribution to breast cancer. We used KLF2, a known MEF2-target gene to estimate the influence of Class IIa HDACs mediate repression. Initially we evaluated the effect of the knock down of HDAC4 in the two cell lines MCF7 and MDA-MB-231. No up-regulation in KLF2 was appreciated with the silencing of solely HDAC4 or other Class IIa deacetylases (Figure 3 A). Interestingly, we noticed the compensatory response between HDAC4 and HDAC5 mRNA levels in ER positive cells (Figure 3 B). Down-regulation of HDAC4 led to an increase of HDAC5.

For this reason, in order to modify Mef2-dependent gene expression, we knocked down three different deacetylases, simultaneously. In MDA-MB-231 cells the selected genes were unaffected by the down-regulation of HDAC4, HDAC5 and HDAC9 (Figure 3 C). A complete different scenario was seen in MCF7 where we were able to notice the rise of the Mef2-dependent transcription, as exemplified by KLF2 up-regulation (Figure 3 C). Furthermore stable HDAC4-GFP overexpression in breast cancer cells caused KLF2 repression only in ER positive cancer cells (Figure 3 D). Conversely the stimulation of Class IIa HDACs nuclear export with the addition of AICAR, a drug that activate AMPK, positively modulate KLF2 expression only in MCF7 cells (Figure 3 D). To understand which deacetylases are involved in the regulation of Mef2 transactivating ability we employed two different siRNAs. Interestingly the decrease of two Class IIa HDACs was sufficient to determine the up-regulation of Mef2 dependent transcription although less potently compared to the down-regulation of three enzymes (Figure 3 E). In summary all the results support that MDA-MB-231 and MCF7 manifest a different repressive ability of Class IIa HDACs.

MEF2 dependent transcription is negatively correlated to aggressive estrogen receptor positive tumors

To verify our approach we scored Class IIa HDACs repressive influence employing a panel of genes, which carry in their proximal promoter the MEF2-binding site

(http://www.broadinstitute.org/gsea/msigdb/index.jsp). Since some MEF2-targets are influenced also by the activity of estrogen receptor we decided to exclude these genes from the analysis. Using Gene Set Enrichment analysis, we initially observed a reduction of MEF2-target genes expression in MCF7 cells compared to MDA-MB-231 cells (Figure 4 A, B). To examine if this correlation is maintained also in human breast cancers, we employed two different data set comparing Grade 3 tumors expressing or not the ER. We selected Grade 3 tumors since both MCF7 and MDA-MB-231 are cell lines derived from metastasis (144). Interestingly there is a negative correlation between Grade 3 ER+ tumors and MEF2-target gene expression (Figure 4 C,D). Next we asked if the levels of MEF2-target genes are affected by the aggressiveness of ER expressing tumors. When compared to Grade 1 and Grade 2, in Grade 3 ER+ breast cancers MEF2-target gene expression is down-regulated and validated in two different datasets (Figure 4 E, F, G). Conversely when the analysis was performed between Grade 1 and Grade 2 ER+ specimens there is no differential correlation (Figure 4 H). These observations suggest a specific down-modulation of MEF2-target genes in relation to the aggressiveness of ER+ breast cancers.

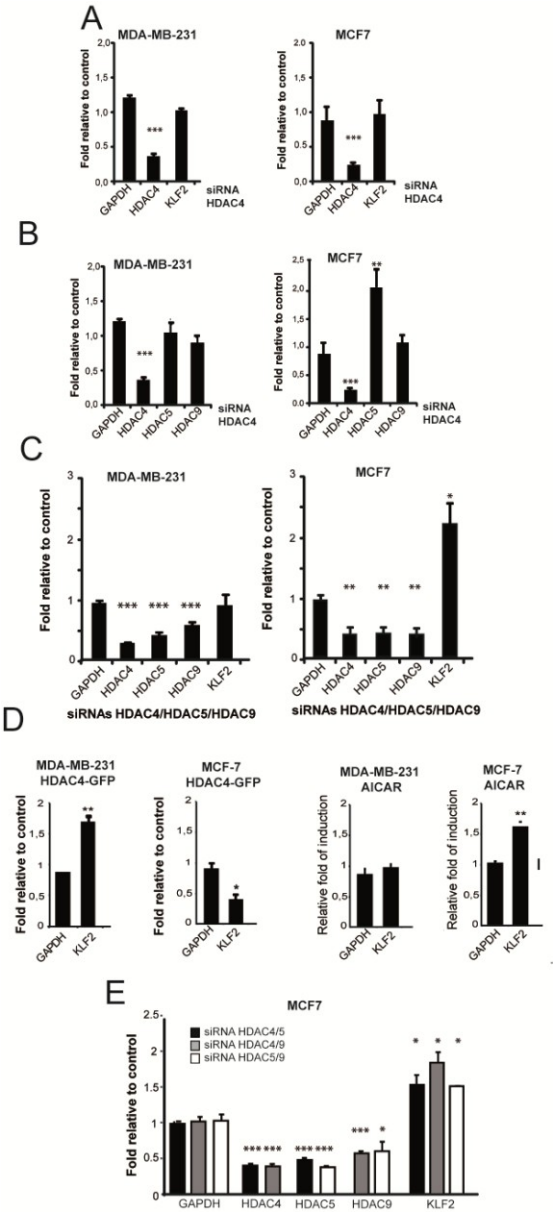


Figure 3 Class IIa HDACs silencing in breast cancer cells is associated with a different response on MEF2 target genes

A) qRT-PCR analysis was performed to quantify mRNAs levels of the MEF2-target gene, KLF2. GAPDH was used as control gene. MCF-7 and MDA-MB-231 cells, transfected with siRNA against HDAC4 were lysed and mRNAs were extracted. The fold induction was calculated as the ratio relative to control siRNA transfected cells.

B) qRT-PCR analysis was performed to quantify mRNAs levels of HDAC4, HDAC5 and HDAC9. GAPDH was used as control gene. MCF-7 and MDA-MB-231 cells, transfected with siRNA against HDAC4 were lysed and mRNAs were extracted. The fold induction was calculated as the ratio relative to control siRNA transfected cells.

C) qRT-PCR analysis was performed to quantify mRNAs levels of the MEF2-target gene,KLF2. GAPDH was used as control gene. MDA-MB-231 and MCF7 cells, were co-transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the same amount of a control siRNA were lysed and mRNAs were extracted. The fold of induction was calculated as the ratio relative to control siRNA transfected cells.

D) qRT-PCR analysis was performed to quantify mRNAs levels of the MEF2-target gene KLF2. GAPDH was used as control gene. MDA-MB-231 and MCF7 cells sably overexpression HDAC4-GFP, or treated with AICAR 100 uM for 24 h, were lysed and mRNA was extracted. The fold induction was calculated as the ratio relative to cells stably expressing GFP or cells treated with vehicle (DMSO)

E) qRT-PCR analysis was performed to quantify mRNAs levels of the MEF2-target gene KLF2. GAPDH was used as control gene. MDA-MB-231 cells, co-transfected with siRNAs against HDAC4, HDAC5 and HDAC4 and HDAC9 or HDAC5 and HDAC9 with the same amount of a control siRNA were lysed and mRNAs were extracted. The fold of induction was calculated as the ratio relative to control siRNA transfected cells

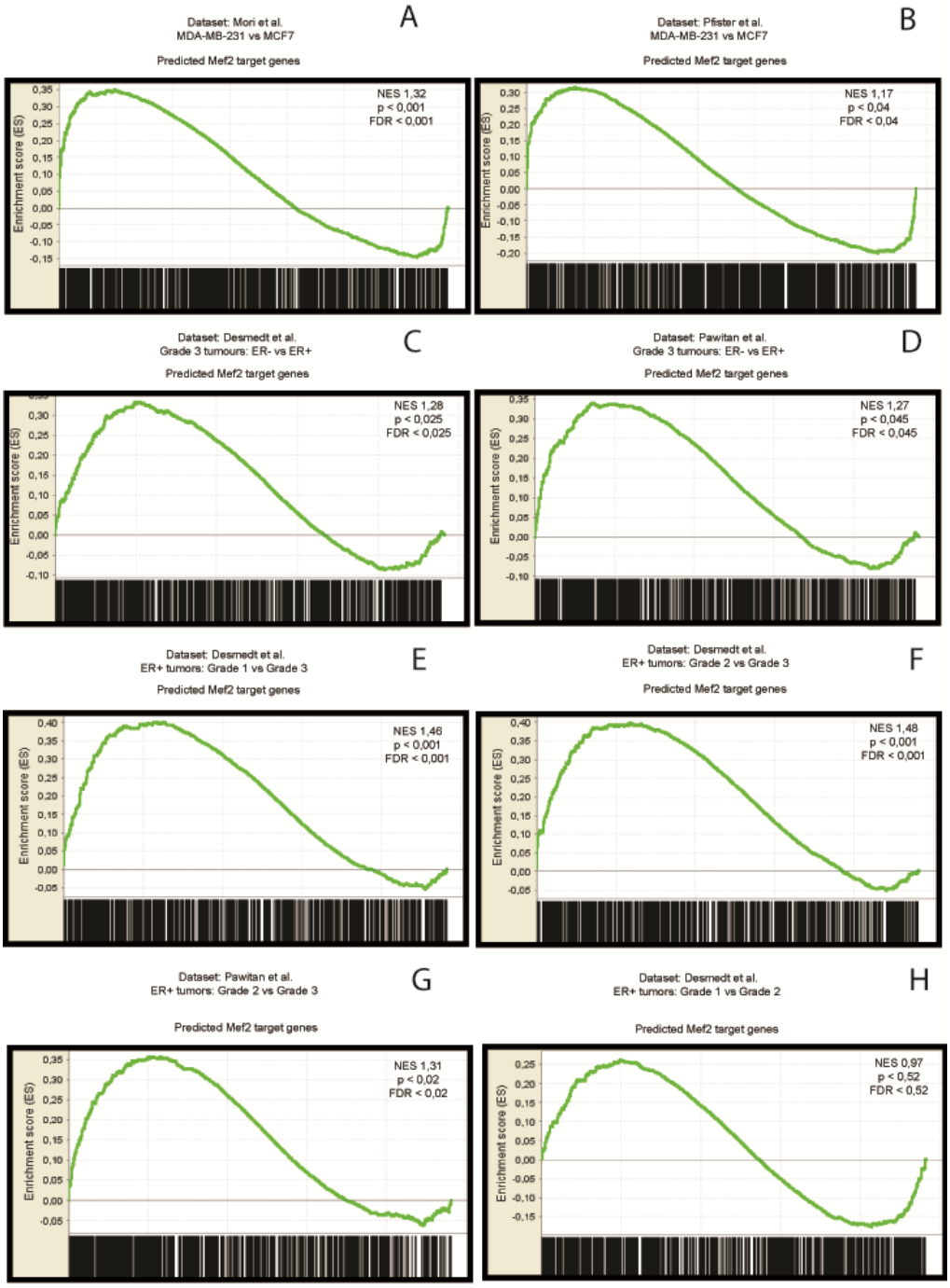


Figure 4 Mef2 dependent transcription is negatively associated with aggressive ER+ breast cancers
Gene Set Enrichment Analysis is a method that establish whether a set of genes shows a difference between two biological states. The Normalized enrichment Score evaluates the degree to which a gene set is associated with a biological condition. Correspondent p values are indicated. A), B) GSEA of putative Mef2 target genes in data set evaluating MDA-MB-231 and MCF7 cells.
C), D) GSEA of putative Mef2 target genes in microarray data comparing human Grade 3 ER- tumors samples to human Grade 3 ER+ tumors.
E)), GSEA of putative Mef2 target genes in microarray data comparing human Grade 3 ER+ tumors to human Grade 1 ER+.
F), G), GSEA of putative Mef2 target genes in microarray data comparing human Grade 3 ER+ tumors to human Grade 2 ER+.
H) GSEA of putative Mef2 target genes in microarray data evaluating of human ER+ Grade 1 to human Grade 2 ER+.

Class IIa HDACs repressive ability is coupled with different deacetylase activity in breast cancer cells

The repressive function of Class IIa depends on their recruitment on chromatin, operated by the partner transcription factors. We supposed that a deficiency in the binding of Mef2 could be a signature of MDA-MB-231 cells. To test this hypothesis we evaluated the interaction between HDAC4 and MEF2D in the two cell lines. Co-immunoprecipitation assay testify that HDAC4 was able to bind MEF2D in both MCF7 and MDA-MB-231 (Figure 5 A) thus excluding alterations in the binding capability. Class IIa HDACs repressive influence depends on the recruitment of an enzymatically active repressive complex composed by SMRT/NCoR-HDAC3 on target promoters. After gel filtration of cellular extracts overexpressing HDAC4, it has been described an enzymatically active complex of a molecular weight above 0,66 MDa (9). Hence, we investigated if also endogenous HDAC4 could be isolated in high molecular weight and whether differences could be appreciated between the two cell lines. In Figure 5 B are shown the immunoblots for the different fractions probed for HDAC4, MEF2D and HDAC3. Overall the pattern is similar in both MCF7 and MDA-MB-231 cells, with only a limited amount of HDAC4 and MEF2D visualized in heavy fraction above 0,66 MDa. Conversely HDAC3 is entirely found in above 0,66 MDa. To confirm that HDAC4 could form a repressive complex at high molecular weight, the different fractions were immunoprecipitated for HDAC4 and next visualized, after immunoblotting, the amount of MEF2D bound to HDAC4 in the different fractions. We initially performed the experiment in MDA-MB-231 cells since they manifest a reduced Class IIa mediated gene repression. In Figure 5B is shown that HDAC4 is able to bind MEF2D forming a complex of heavy-molecular weight above 0,66 MDA. Similar result was observed in MCF7 cells (data not shown). Hence, impairments in the formation of the HMW complex cannot explain the lack of KLF2 up-regulation in MDA-MB-231 cells, after HDAC4, 5 and 9 silencing.

Although the HMW complex could be similarly assembled in the two cell lines, its repressive activity could be altered in MDA-MB-231 cells. To prove this assumption, HDAC4 was immunoprecipitated from the two cell lines and the associated deacetylase activity was scored, using a fluorescent assay. A deacetylase activity was observed in both samples but the amount was markedly reduced in MDA-MB-231 cells, compared to MCF7 (Figure 6 A). The difference was even more impressive considering the amount of the immunoprecipitated HDAC4, markedly higher in MDA-MB-231 (Figure 6 B). Normalization of the enzymatic activity respect ot the amount of immunoprecipitated HDAC4, evidenced five fold difference in HDAC4-associated deacetylase

activity between MCF7 and MDA-MB-231 cells (Figure 6 C). Since HDAC3 is an important player in Class IIa HDACs mediated deacetylase activity we evaluated its expression in the two cell lines. Figure 6 D it is shown that MCF7 cells expressed higher levels of HDAC3.

Taking into account that Mef2 target genes are differentially modulated in breast cancer cells and that MCF7 cells manifest an important Class IIa HDACs mediated repression, we speculated that the modulated Mef2 target gene KLF2 should be less expressed in ER expressing cells. In Figure 6 E we report the relative mRNA levels for the previously tested gene and observed a clear down-regulation in the luminal cell line compared to triple negative cells. All these observations suggest that Class IIa HDACs mediated transcriptional repression of MEF2-dependent transcription plays an important role in ER positive cancer cells.

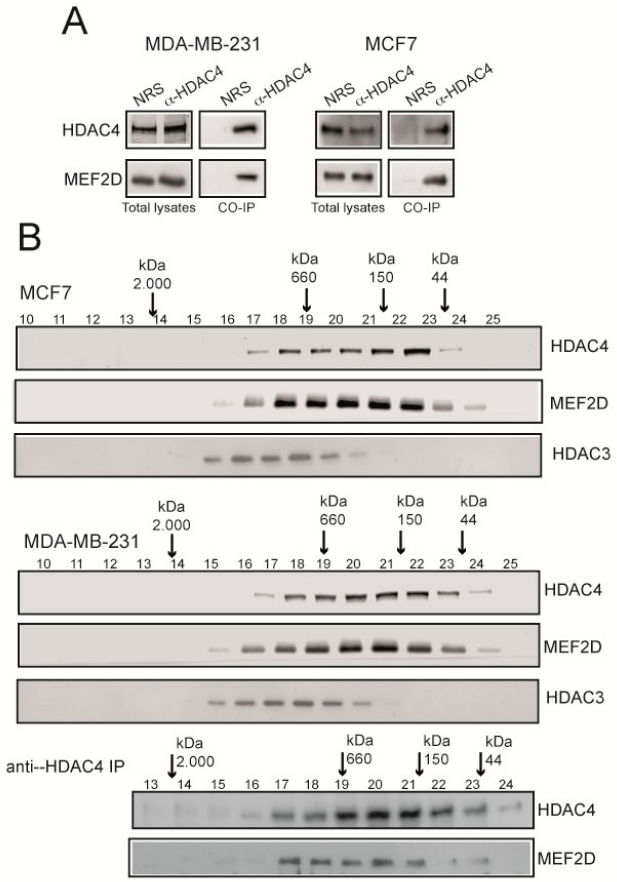


Figure 5. Analysis of the multiproteins complex containing HDAC4 in breast cancer cell lines.
A) Cellular lysates from MDA-MB-231 and MCF-7 cells were immunoprecipitated using an anti-HDAC4 antibody or normal rabbit serum (NRS). Immunocomplexes were next probed with anti-MEF2D or anti-HDAC4 antibodies, as indicated. A fraction of the lysates before immunoprecipitation was used as input (Total lysates).
B) Cellular lysates from MDA-MB-231 and MCF-7 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 performed with the anti-MEF2D or anti-HDAC4 antibodies. Arrows indicate the elution positions of molecular weight standards.

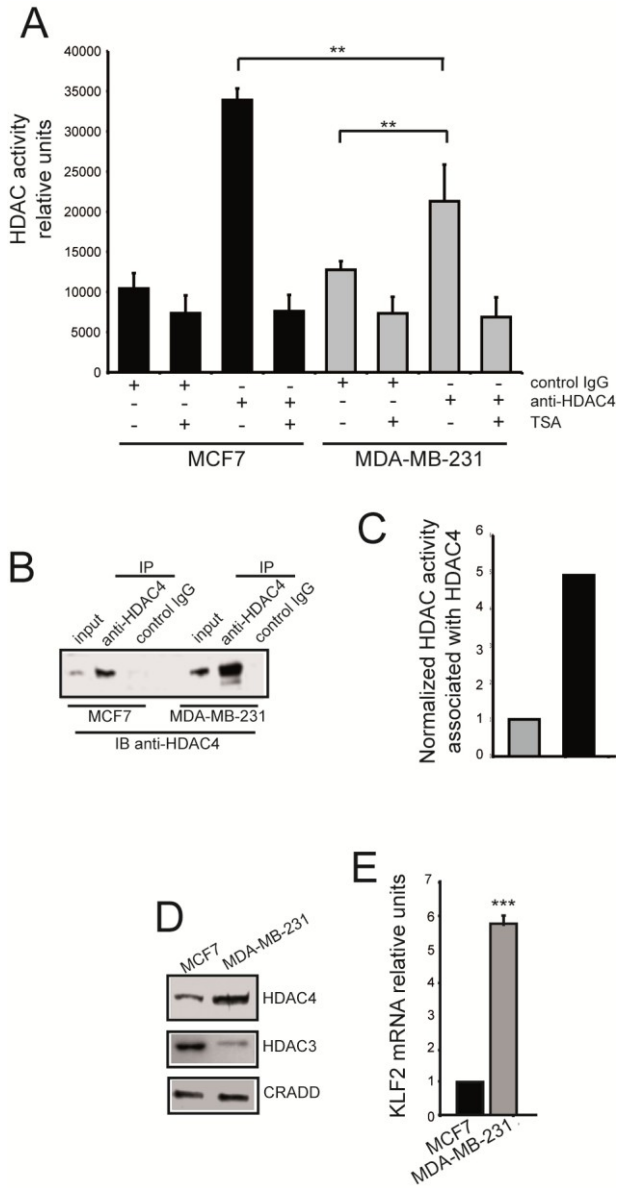


Figure 6. Analysis of HDAC4 repressive influence in breast cancer cell lines.

A) Cellular lysates from MDA-MB-231 and MCF-7 cells were immunoprecipitated using an anti-HDAC4 antibody or control rabbit immunoglobulins (IgG). After several washes immunocomplexes were incubated with the Fluor de Lys® substrate. TSA was used at 40μM final concentration. Data are from three independent experiments.

B) A fraction of the immunoprecipitations analyzed for the deacetylase activity was separated on a SDS/PAGE gel electrophoresis and after immunoblotting HDAC4 was visualized using anti-HDAC4 antibody.

C) Densitometric analysis was performed on the immunoblot showed in figure B to normalize HDAC activity to the amount of HDAC4 purified from the two cell lines.

D) Cellular lysates generated from MCF-7 and MDA-MB-231 cell lines were subjected to immunoblot analysis using the specific antibodies as indicated. CRADD was used as loading control.

E) qRT-PCR analysis was performed to compare KLF2 mRNAs levels between MCF-7 and MDA-MB-231 cells. The fold induction was calculated as the ratio relative to KLF2 mRNA levels in MCF-7 cells..

Class IIa HDACs regulate survival of MCF7 cells

Described the influence of Class IIa HDACs on Mef2 dependent transcription we next considered their effect on cancer cells growth. Knock-down of HDAC4, HDAC5 and HDAC9 caused an inhibition of cell proliferation in MCF7 (Figure 7 A), this effect not observed with the down-regulation of only one deacetylase (Data not shown). Given that alterations in cell cycle were not observed after cytofluorimetric analysis of MCF7 cells treated with three different siRNAs (Figure 7 B), we investigated the appearance of cell death. An increase in trypan blue positive cells was evident in MCF7 cells silenced for HDAC4, HDAC5 and HDAC9 (Figure 7 C). We also evaluated additional markers of cell death. We scored the re-localization of SMAC, an intermembrane mitochondrial protein, as a sign of mitochondria outer membrane permeabilization (MOMP), and the release of HMGB1 in the cytoplasm (Figure 7 D). In both cases cell death after silencing of the three different deacetylases was confirmed by analysis of these two markers (Figure 7 E).

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

Nur77/NR4A1 is an orphan nuclear receptor, induced by Mef2 transcription factors, that plays a central role in the development of T cells causing apoptosis of auto-reactive T cells (96). To gain insight in the pro-survival activity of Class IIa HDACs we analyzed the expression of the NR4 orphan nuclear receptor family members after the knock-down of the three different deacetylases. Interestingly, only Nur77/NR4A1 mRNA was up-regulated after silencing of HDAC4, HDAC5 and HDAC9 (Figure 8 A). Similarly to the other Mef2 target genes, in MDA-MB-231 cells perturbations in the levels of NR4 family members were absent, after transfection of the three different siRNAs (Figure 8 B). Next we compared the amount of mRNA levels of these receptors between MCF7 and MDA-MB-231. Contrary to KLF2, MCF7 cells display higher levels of Nurr77, while Nurr1 and Nor1 where similar (Figure 8 C). This was not surprising since Nur77 is also a transcriptional target of the estrogen receptor (145). Finally we explored whether by modulating Nur77 levels we could induce cell death in MCF7 cells. Nur77-GFP or GFP alone were transfected in MCF7 cells and induction of cell death was scored by observing SMAC release from mitochondria. As exemplified by immunofluorescence and by quantitative analysis (Figure 8 D, E) Nur77 overexpression caused an increase in cell death. This was also supported by the increase of processed pro-caspase 3 observed in Nur77-GFP transfected cells (Figure 8 F).

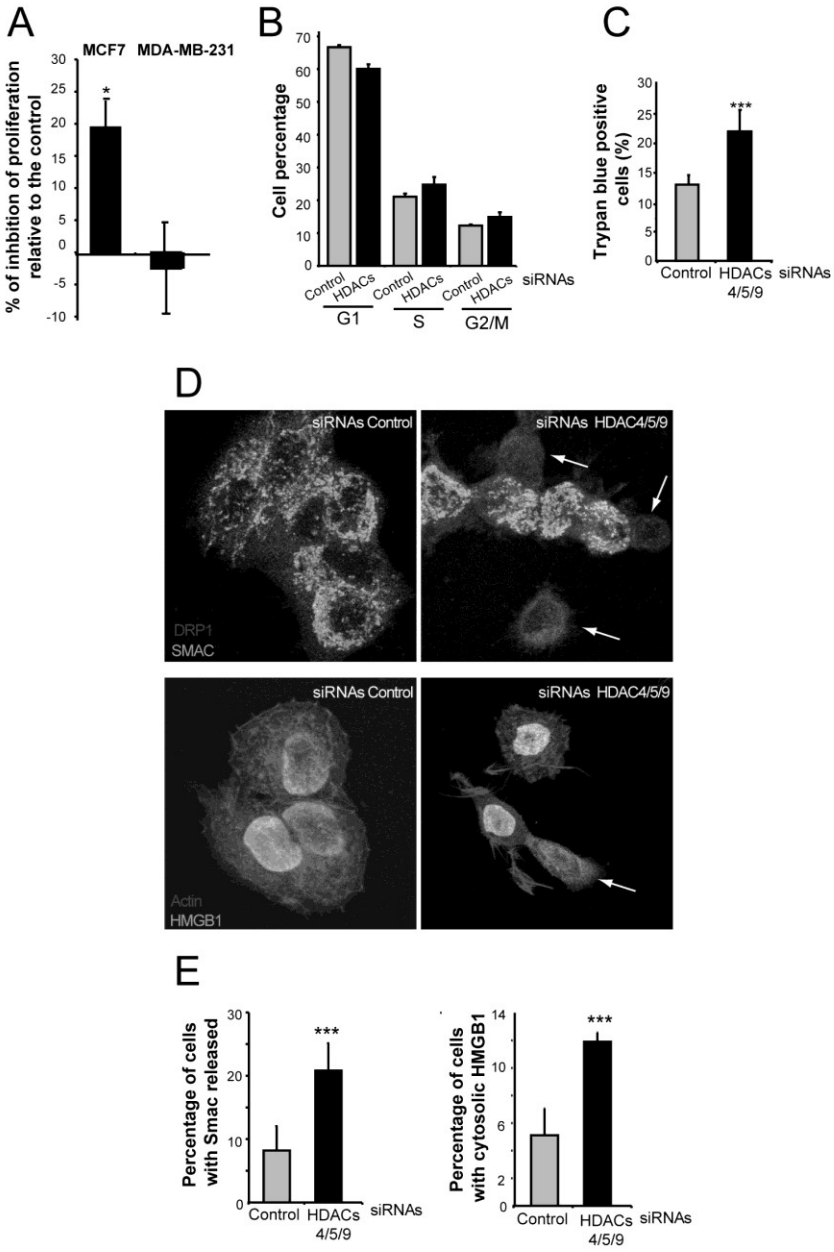


Figure 7. Class IIa HDACs control MCF-7 cell survival

A) MCF-7 and MDA-MB-231 cells were transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the control siRNA. 48 h later cells were counted. Data are presented as percentage of inhibition of the triple siRNAs relative to control.

B) MCF-7 cells were transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the control siRNA. 48 h later cells were fixed and cell cycle profiles were assessed after staining with PI, by FACS analysis. (means +SD, n=3).

C) MCF-7 cells were transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the control siRNA. 48 h later cell dead was analyzed after Trypan blue staining.

D) Confocal pictures illustrating the subcellular localization of SMAC and of HMGB1 in MCF-7 cells transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the control siRNA. 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.

E) Quantitative analysis of SMAC and HMGB1 localization as described in (D).

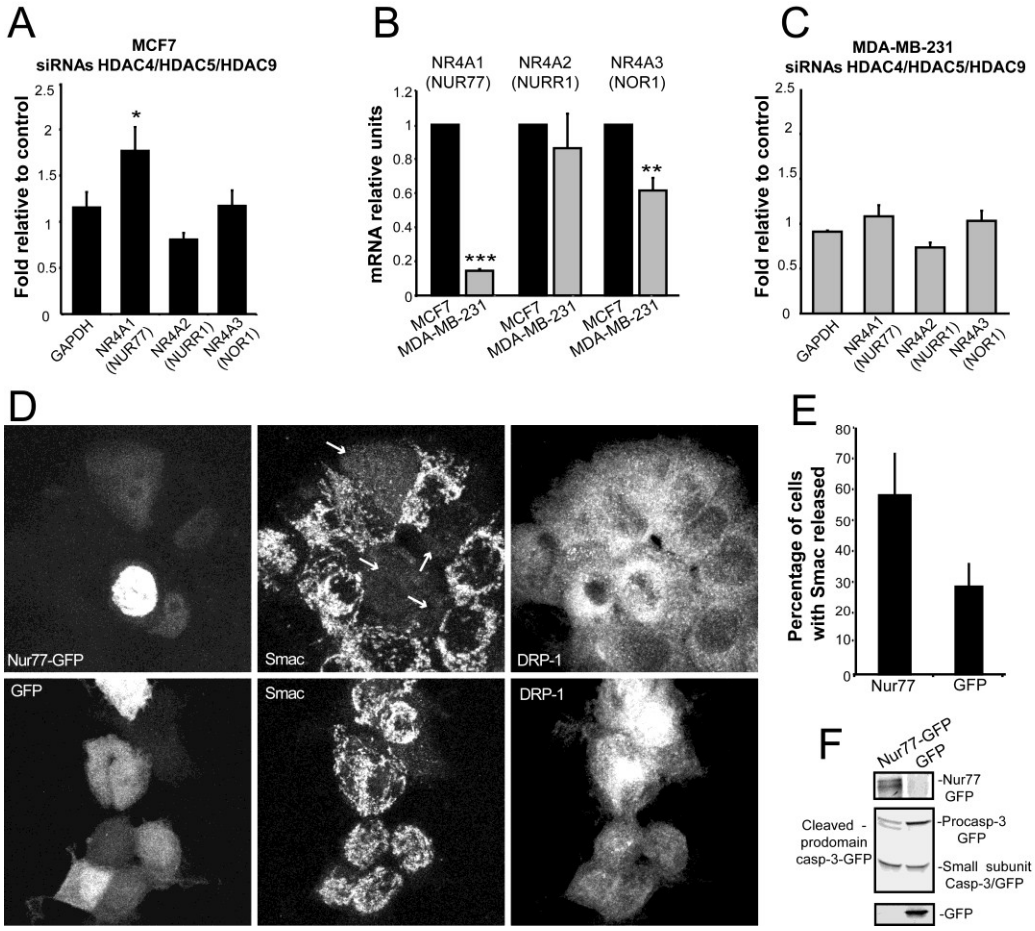


Figure 8. Class IIa HDACs repress NR4A1/NuR77 expression in MCF-7 cells

A) qRT-PCR analysis was performed to quantify mRNAs levels of the Nur77 family members, *NR4A1, NR4A2* and *NR4A3*. *GAPDH* was used as control gene. MCF-7 cells, co-transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the same amount of a control siRNA were lysed and mRNAs were extracted. Samples. The fold of induction was calculated as the ratio relative to control siRNA transfected cells.

B) qRT-PCR analysis was performed to compare *NR4A1, NR4A2* and *NR4A3* mRNAs levels between MCF-7 and MDA-MB-231 cells.. The fold induction was calculated as the ratio relative to *NR4As* mRNA levels in MCF-7 cells.

C) qRT-PCR analysis was performed to quantify mRNAs levels of the Nur77 family members, *NR4A1, NR4A2* and *NR4A3*. *GAPDH* was used as control gene. MDA-MB-231 cells co-transfected with siRNAs against HDAC4, HDAC5 and HDAC9 or with the same amount of a control siRNA were lysed and mRNAs were extracted.. The fold of induction was calculated as the ratio relative to control siRNA transfected cells.

D) Confocal pictures illustrating the subcellular localization of SMAC in MCF-7 cells transfected with NR4A1/Nur77-GFP or with GFP alone. 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 (D).

F) Caspase-3/GFP together with Nur77-GFP or GFP alone were transiently expressed in MCF-7 cells. After 24 h from transfection cell lysates were generated and subjected to immunoblotting using the anti-GFP antibody.

Class IIa HDACs specific inhibitor promotes cell death in MCF7 but not in MDA-MB-231 cells.

In order to recapitulate the effects of the multiple knock-down of Class IIa HDACs and to verify the effect of silencing, we decided to use an inhibitor of Class IIa HDACs. Recently it has been discovered that the compound, N-Lauroyl-D/L-Phenyl-Alanine, characterized for a specific effect over Class IIa enzymes, with an *in vitro* IC_{50} value around 20 μM, respect to Class I and Class IIb HDACs (146). Addition of this inhibitor to the medium of breast cancer cells at 100μM concentration up-regulated the expression of the Mef2 target genes: KLF2 and NR4A1/NUR77, thus recapitulating the results obtained with the silencing of different Class IIa HDACs. Again similarly to the triple silencing a panel of Mef2 target genes extrapolated from the gene set used for enrichment analysis was preferentially up-regulated in MCF7 (Figure 9 A). NR4A1 was modulated in both cell lines but the entity of up-regulation was clearly different. Furthermore N-Lauroyl-D/L-Phenyl-Alanin elicited a growth inhibitory effect and cell death only in the luminal cell line as evidenced by trypan blue counting and MCF7 cells (Figure 9 B, C)

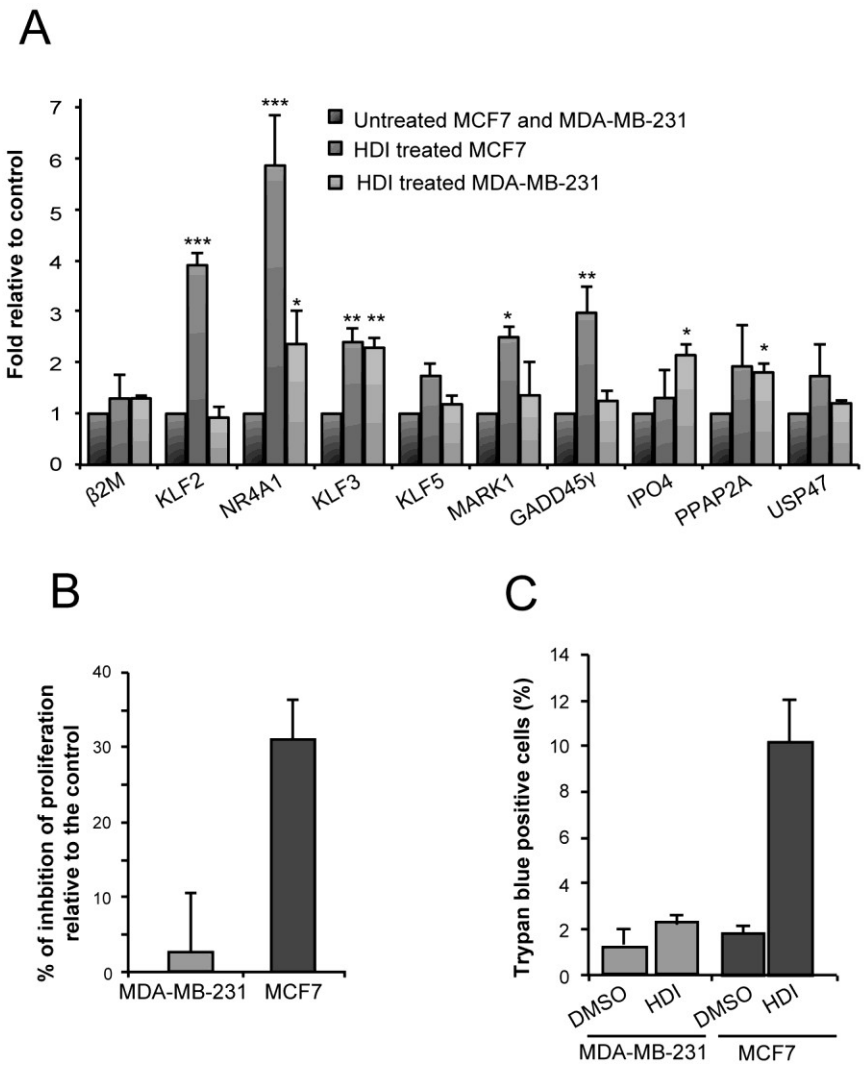


Figure 9. Inhibition of Class IIa HDACs on breast cancer cells

A) qRT-PCR analysis was performed to quantify mRNAs levels of the MEF2-target genes, KLF2, NR4A1, KLF3, KLF5, MARK1, GADD45gamma, IPO4, PPAP2A, USP47. B2M was used as control gene. MCF-7 and MDA-MB-231 cells were treated with 100 uM HDAC inhibitor N-Lauroyl-N-Phenyl-Alanine or with vehicle (DMSO) for 48 h and then cells were lysed and mRNAs were extracted. The fold induction was calculated as the ratio relative to control vehicle treated cells.

B) MCF-7 and MDA-MB-231 cells were treated with 100 uM HDAC inhibitor N-Lauroyl-N-Phenyl- Alanine or with vehicle (DMSO) for 48 h and then cells were collected. Data are presented as percentage of inhibition of the treated cells relative to control.

C) MCF-7 and MDA-MB-231 cells were treated with 100 uM HDAC inhibitor N-Lauroyl-N-Phenyl- Alanine or with vehicle (DMSO) for 48 h and then cells were collected. Cell death was evidecnced with Trypan blue staining.

Discussion

HDAC4 heterogeneity in breast cancer

In this work we have analyzed mutational status, expression levels and localization of HDAC4 in a panel of different breast cancer cells that mimic the heterogeneity of human cases. We were unable to notice a correlation between HDAC4 expression and a specific cancer subtype and this variability is also observed in immunohistochemistry of human samples. The heterogeneous pattern of expression of HDAC4 could be buffered by changes in other Class IIa HDACs members, making a correlation difficult to be observed. For example in the luminal cell line ZR-75-1 higher levels of HDAC4 can compensate lower amounts of HDAC7 and HDAC9 compared to MCF7 and T47D cells. Moreover this variability could also be the result of specific alterations in miRNAs that can control the expression of these enzymes, as already observed in other contexts (18,122). However we were able to notice an association between Class IIa HDACs mRNA expression levels and a decrease in the overall survival only in luminal breast cancers suggesting a selective requirement of these epigenetic regulators in a restricted subset of human breast tumors. Conversely no relationship has been observed in estrogen receptor negative cells. Curiously we noticed an alteration of HDAC4 nuclear import in the MDA-MB-468. This can be the result of specific genetic lesions affecting regulators of Class IIa HDACs shuttling or could represent an adaptation to alter Class IIa HDACs activity, beyond the control their expression. Alteration of class IIa HDACs subcellular localization could be frequent in breast tumors, thus explaining the great difference observed in HDAC4 localization between cancer cell lines and tumor samples.

Repressive ability of Class IIa HDACs in breast cancer cells

Class IIa HDACs are important epigenetic regulators when resident in the nucleus, but they can also play additional roles in the cytoplasm modulating different signaling pathways such as Wnt or Hif-1 (137,147) Given the association between the expression of these enzymes and a bad prognosis, we initially explored whether changes in the repressive influence could be coupled with their repressive ability. We concentrated our analysis on Mef2 dependent transcription, since this family of transcription factors is the favorite partner of Class IIa HDACs. Our analysis evidenced the existence of redundant and compensatory mechanisms that buffer Class IIa HDACs expressional changes to preserve gene expression from fluctuations, in the luminal cell line MCF7. This effect

was also reported in different contexts such as liver or skeletal muscles (17,27). Moreover we were able to selectively modulate KLF2 only in ER+ cells while no changes on this Mef2 target gene was appreciated in MDA-MB-231 cells. Class IIa HDACs regulation of Mef2 transcription seems to be selectively associated with luminal cancer cells as exemplified by an extensive GSE analysis using a Mef2-dependent signature. Interestingly this signature is repressed in aggressive estrogen receptor positive tumors, indicating the specific need to down-modulate Mef2 genetic program with grade progression, phenomenon not observed in ER- specimens. All these considerations converge to identify Class IIa HDACs as important entities in ER+ tumorigenesis.

Class IIa HDACs regulate survival in MCF7 cells

GSE analysis not only delineates the repressive function of Class IIa HDACs but also suggest which subtype of breast cancer could be affected by targeting these repressors. In fact the down-regulation of HDAC4, HDAC5 and HDAC9 influences the survival in MCF7 cells but not in MDA-MB-231 cells. This effect can be explained with the increased expression of the pro-apoptotic gene NUR77/NR4A1, a Mef2 target gene involved in the regulation of apoptosis (96,126). Previous works also identified this gene as an apoptotic inducer in cancer cells. Two principle mechanisms have been proposed to explain the pro-apoptotic effect of NUR77. A direct mechanism, after translocation of this protein to mitochondria or an indirect effect through its activity as transcription factor and the up-regulation of other pro-apoptotic factors (148,149). In our experiments Nur77 over-expression was able to cause cell death thus confirming its pro-apoptotic role and that by affecting Class IIa HDACs function in MCF7 cells apoptosis can arise. NUR77 expression is higher in the luminal cells compared to MDA-MB-231 indicating that Mef2s are in concern with other transcription factors and cooperate with them to promote context specific expression of their targets (150). However, we cannot exclude the contribution of other Mef2 target genes in the regulation of cell survival or additional genetic programs, beyond Mef2 activation, that are modulated after the knock-down of multiple Class IIa HDACs.

Inhibition of Class IIa HDACs is coupled to altered gene expression and to cell death only in MCF7 cells

Different inhibitors that target HDACs are available and some of the are already in clinic for the treatments of certain neoplasias. However most of them exert a preferred action over Class I enzymes. Conversely relatively few compounds are disposable to target Class II and specifically Class IIa. Some reports described the action of one compound that, acting of Class IIa enhances

their repressive ability favoring the interaction with HDAC3 (11). We used a recently described drug N-Lauroyl-Phenyl-Alanine, characterized for the marked inhibition of HDAC7 compared to Class I and Class IIb, by in vitro studies. We decided to use this inhibitor to recapitulate and strengthen the effect of silencing HDAC4, HDAC5 and HDAC9. Inhibition of Class IIa HDACs was characterized by a clear the up-regulation of the MEF2-dependent transcription in MCF7, but not in MDA-MB-231 cells. The expression of several MEF2-targets was clearly induced in MCF7 cells, whereas such response was less dramatic in MDA-MB-231 cells. Several MEF2-targets genes were modulated with a strongest effect on NR4A1/NUR77. The response on gene expression was also linked to the stimulation of cell death in ER+ cells. In summary these results point to Class IIa HDACs as potential therapeutic targets for a selective group of breast tumors.

Perspectives

In summary in this thesis we have characterized Class IIa HDACs function in the breast cancer context demonstrating their association with ER+ tumors. We specifically focused on their regulatory effect over Mef2 transcription factors, central players in the establishment of a correct differentiation program in different contexts. However Mef2 role in breast cancer has not yet been described. The specific modulation of Class IIa HDACs on this pathway could represent the necessity of a specific lineage of cancer cells during the course of the disease. Unluckily the relatively lack of models for progression of ER+ positive tumors renders difficult to address at what stage Class IIa HDACs function is required. It would be interesting to carefully depict the recruitment of these enzymes on chromatin, through ChIP-seq technology, in tumors with a different degree of aggressiveness, to detect specific changes, as already described for the ER (151).

Additional work

During the three year PhD period I've been focused in the characterization of Class IIa HDACs regulation and function. In parallel to the investigation of the role played by these repressors in breast cancer, I've been also involved in the examination of Class IIa HDACs control.

Specifically we have shown that Hdac4 levels are hold in check by the ubiquitin proteasome system. Ubiquitination is a signal dependent process that allows the selective degradation of proteins. We noticed that the lack of growth factors stimulation promotes the destruction of Hdac4 via the UPS. Interestingly this regulation is observed in untransformed cells, of both epithelial and mesenchymal origin, while is lost in cancer cells. Hdac4 presents specific sequences called PEST, that can act as phosphodegrons when phosphorylated. Phosphodegrons allows the interactions with E3 ligase enzymes to favor ubiquitination. The Hdac4 mutant S298D, that mimics a constitutive phosphorylation in PEST sequence, in highly ubiquitinated, also in the presence of growth factors. The Serine 298 is involved also in the regulation of Hdac4 localization. In fact previous observations described that this mutant is usually confined in the cytoplasm (37). Interestingly inhibition of the proteasome using MG132 promotes a transient increase in Hdac4 S298D nuclear staining. Moreover treatment with leptomycin b, inhibitor of the nuclear export, in cells pretreated with MG132 induces its nuclear accumulation while leptomycin b alone is not effective. These observations suggest that Hdac4 S298D is able to enter in the nuclear compartment and where it becomes prone to degradation. These results will be not discussed in this thesis, since the work has been already published (see the enclosed published manuscript).

These observations add further complexity in the regulation of Class IIa HDACs. Interestingly the PEST sequence of Hdac4 is conserved among Hdac5 and Hdac9, indicating the possibility of a common regulation for these deacetylases. Starvation induced degradation of Hdac4 is located in the nucleus, probably eliminating the repressive fraction of this enzymes, while the cytoplasmic pool is conserved to be used in the case of changes from the external environment.

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Ubiquitin-dependent degradation of HDAC4, a new regulator of random cell motility

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ABSTRACT **HDAC4 (histone deacetylase 4) belongs to class IIa of histone deacetylases, which groups important regulators of gene expression, controlling pleiotropic cellular functions. Here we show that, in addition to the well-defined nuclear/cytoplasmic shuttling, HDAC4 activity is modulated by the ubiquitin–proteasome system. Serum starvation elicits the poly-ubiquitination and degradation of HDAC4 in nontransformed cells. Phosphorylation of serine 298 within the PEST1 sequence plays an important role in the control of HDAC4 stability. Serine 298 lies within a glycogen synthase kinase 3β consensus sequence, and removal of growth factors fails to trigger HDAC4 degradation in cells deficient in this kinase. GSK3β can phosphorylate HDAC4 in vitro, and phosphorylation of serine 302 seems to play the role of priming phosphate. We have also found that HDAC4 modulates random cell motility possibly through the regulation of KLF2 transcription. Apoptosis, autophagy, cell proliferation, and growth arrest were unaffected by HDAC4. Our data suggest a link between regulation of HDAC4 degradation and the control of cell motility as operated by growth factors.**

Monitoring Editor
William P. Tansey
Vanderbilt University

Received: Jul 7, 2010
Revised: Nov 12, 2010
Accepted: Nov 15, 2010

INTRODUCTION

Lysine acetylation is emerging as a widespread posttranslation modification (PTM) involved in the regulation of several cellular functions (Choudhary *et al.*, 2009). Histone deacetylases (HDACs) are an assorted family of nuclear and cytoplasmic enzymes involved in reversing this PTM. The HDAC family can be clustered into distinct classes based on sequence and structural homologies to yeast enzymes Rpd3, Hda1, and Sir2 (Yang and Grégoire, 2005). HDAC4 is a member of class IIa, which is characterized by homologies with yeast Hda1, nuclear cytoplasmic shuttling, and heterogeneous levels of expression in various tissues. Similarly to other class IIa members, HDAC4 contains intrinsic nuclear import and export sequences on which converge distinct signaling pathways to modulate its repressive influence (Yang and Seto, 2008).

HDAC4 impinges on multiple and apparently contradictory cellular fates, including differentiation, apoptosis, survival, cell growth, and proliferation (Paroni *et al.*, 2004; Vega *et al.*, 2004; Bolger and Yao, 2005; Backs *et al.*, 2006; Paroni *et al.*, 2007; Wilson *et al.*, 2008; Cadot *et al.*, 2009; Chen and Cepko, 2009). Not surprisingly, multiple levels of regulation mirror this exaggerated versatility. The control of HDAC4 nuclear cytoplasmic shuttling seems to be the master option for modulating its activity (Paroni *et al.*, 2004; Ago *et al.*, 2008). As reported in several studies, HDAC4 nuclear cytoplasmic shuttling is under the control of different kinases and phosphatases, in cooperation with 14–3-3 proteins. Phosphorylation/dephosphorylation cycles efficiently and rapidly couple the repressional activity of class IIa HDACs to environmental signals (Grozinger and Schreiber, 2000; Wang *et al.*, 2000; McKinsey *et al.*, 2001; Martin *et al.*, 2008; Paroni *et al.*, 2008; Yang and Seto, 2008).

HDAC4 activities can be modulated by additional strategies. In colon, HDAC4 is massively expressed in the proliferative compartment and is down-regulated during intestinal differentiation (Wilson *et al.*, 2008). In muscle, denervation modulates HDAC4 expression (Cohen *et al.*, 2009; Tang *et al.*, 2009).

Despite changes in HDAC4 levels having been observed in different situations, the mechanisms responsible for such fluctuations are poorly defined. Sp1 and Sp3 transcription factors regulate HDAC4 transcription, but how this control integrates with cellular signaling networks is unclear (Liu *et al.*, 2006). In addition, posttranscriptional strategies to modulate HDAC4 mRNA levels also exist;

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-07-0616) on November 30, 2010.

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Abbreviations used: Gas2, growth arrest specific gene 2; GSK3β, glycogen synthase kinase 3β; HDAC, histone deacetylase; KLF2, Krüppel-like factor 2; LC3, light chain 3; MEF2, myocyte-enhancer factor 2; PCNA, proliferating cell nuclear antigen; PTM, posttranslation modification; QRT-PCR, quantitative real-time PCR.

for example, miR-1 contributes to repress HDAC4 levels during muscle differentiation (Sun *et al.*, 2010).

Here we have investigated the expression levels of HDAC4 in response to growth factors. We have discovered that growth factor deprivation elicits poly-ubiquitination and proteasome-mediated degradation of HDAC4. Serines at position 298 and 302 and the kinase glycogen synthase kinase 3β (GSK3β) seem to be important elements of the signaling pathway governing HDAC4 stability. Finally, we have explored which among the different cellular responses under serum regulation, such as autophagy, apoptosis, cell cycle, and motility could be influenced by HDAC4. Our results suggest that HDAC4 regulates random cell motility. Overall this study discovers a new mechanism of HDAC4 regulation and provides a link between HDAC4 degradation and the decline of cell motility elicited by growth factor withdrawal.

RESULTS

Growth factors modulate HDAC4 expression levels in normal breast cells

In the immortalized, nontransformed mammary epithelial cell line MCF-10A, proliferation is modulated by exogenously added growth factors. We used MCF-10A cells to evaluate the effect of growth factors on the expression levels of HDAC4. Cells were grown for 24 h in complete medium; next serum and additional growth factors were removed. Growing MCF-10A cells for another 48 h in low serum (0.5% fetal bovine serum [FBS]) promotes exit from the cell cycle, as evidenced by proliferating cell nuclear antigen (PCNA) down-regulation (Figure 1A). HDAC4 levels were similarly down-regulated. Next we evaluated the effect of different medium components on HDAC4 levels. Addition of hydrocortisone, insulin, and cholera toxin was sufficient to sustain HDAC4 and PCNA expression in low serum. Curiously some increase in HDAC4 but not in PCNA levels can be seen after supplementing low serum medium with cholera toxin and hydrocortisone (Figure 1A). Re-addition of growth factors to MCF-10A–starved cells promoted cell proliferation and HDAC4 expression (unpublished data).

To evaluate changes in the amount of HDAC4 transcripts by low serum, we used quantitative real-time PCR (QRT-PCR) analysis. RNA was extracted from growing MCF-10A cells (cultured for 24 h in complete medium) or from cells cultured for different times in 0.5% FBS. As shown in Figure 1B, HDAC4 mRNA levels were unaffected by growth factor removal.

Having excluded changes in mRNA levels, HDAC4 down-regulation after serum starvation could be the consequence of proteolysis or of translation control. To discriminate between these possibilities, MCF-10A cells were cultured in low serum in the presence of the proteasome inhibitor MG132. Figure 1B confirms that serum starvation and elimination of growth factors elicit the down-regulation of HDAC4, already evident 24 h after the removal. Growing cells for an additional 24 h in low serum further decreased HDAC4 levels. This down-regulation was efficiently prevented by the presence of MG132.

To understand whether HDAC4 is also degraded via the ubiquitin–proteasome system in the presence of growth factors, MCF-10A cells were grown for 48 or 72 h in complete medium in the presence or not of MG132. Figure 1D shows that, in this case, HDAC4 levels were not increased after proteasomal inhibition.

Cancer cells also can proliferate in the presence of limited amounts of growth factors. Interestingly, HDAC4 levels were unaffected in the breast cancer cell line MCF-7, cultured in low serum conditions (Figure 1E). Similar results were obtained with the glioblastoma cell line U87MG. Hence degradation of HDAC4, as elicited by the absence of growth factors, is a prerogative of nontransformed cells.

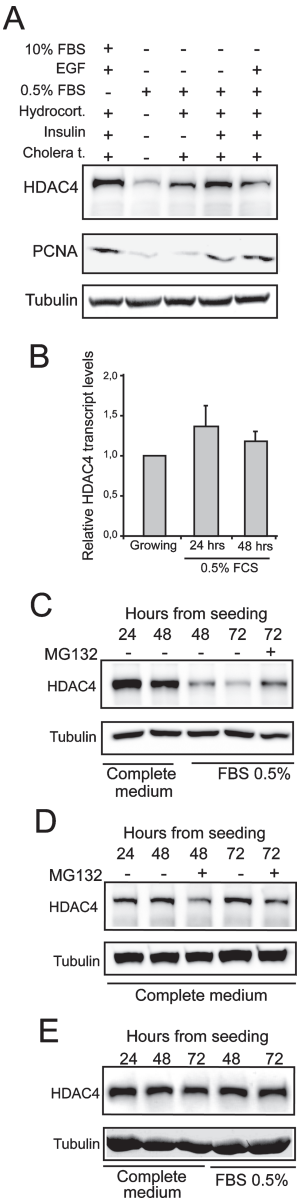

FIGURE 1: Proteasomal-dependent degradation of HDAC4 upon growth factor removal. (A) MCF-10A cells were grown in complete medium for 24 h (lane 1). Next cells were incubated with different media, as indicated, for another 48 h. Cellular lysates were generated and subjected to immunoblot analysis using the specific antibodies. Tubulin was used as loading control. (B) Regulation of HDAC4 mRNA expression by low serum. MCF-10A cells were grown in complete medium for 24 h or in low serum for an additional 24 and 48 h. RNA was extracted, and QRT-PCR analysis was performed to quantify HDAC4 mRNAs. Samples were normalized to GAPDH (means ± SD, n = 4). (C) MCF-10A cells were grown in complete medium for 24 h or in low serum for an additional 24 or 48 h. MG132 (2.5 μM) was added to cells 24 h before cell lysis. Cellular lysates were subjected to immunoblot analysis using anti-HDAC4 antibodies. Tubulin was used as loading control. (D) MCF-10A cells were grown in complete medium for the indicated times. When used, MG132 (2.5 μM) was added to cells 24 h before cell lysis. Cellular lysates were generated and subjected to immunoblot analysis using anti-HDAC4 antibodies. Tubulin was used as loading control. (E) MCF-7 breast cancer cells 24 h after seeding were maintained in 10% FBS (complete medium) or grown in low serum (0.5% FBS) for the indicated times. Cellular lysates were generated and subjected to immunoblot analysis using anti-HDAC4 antibodies. Tubulin was used as loading control.

Proteasome-dependent degradation of HDAC4 in NIH3T3 cells during growth arrest induced by serum starvation

To confirm that proteasome-dependent degradation of HDAC4 in response to serum deprivation is not limited to MCF-10A cells, we used NIH3T3 murine fibroblasts. Initially we evaluated HDAC4 levels in cells grown for 24 h in 10% FBS (growing cells) and following 24 or 48 h of culture in low serum conditions. MG132 was used to evaluate the contribution of the ubiquitin-dependent degradation. Figure 2A demonstrates that serum starvation also promotes proteasomal degradation of HDAC4 in NIH3T3 cells. Similarly to what was observed in MCF-10A cells, HDAC4 was selectively targeted to the proteasome only when serum was removed. In fact, addition of MG132 to actively proliferating cells does not influence HDAC4 levels (Figure 2B).

We also analyzed whether serum starvation modulates the subcellular localization of HDAC4 and whether interfering with HDAC4 degradation could influence its nuclear accumulation. In growing cells, HDAC4 shows a cytoplasmic localization in the majority of the cells. A pancellular localization can be observed in a limited number of cells, whereas rare cells accumulate HDAC4 in the nucleus (Figure 2C). In serum-starved cells, HDAC4 levels were clearly reduced. When we performed a quantitative analysis (unpublished data), however, the subcellular localization was unchanged compared to growing cells. Treatment of serum-starved cells with leptomycin B, an inhibitor of CRM1-mediated nuclear export, elicited the nuclear accumulation of HDAC4 (Figure 2C) similarly to growing cells (unpublished data). This result demonstrates that HDAC4 is subjected to nuclear cytoplasmic shuttling, also under serum starvation. Incubation with MG132 increased the signal for HDAC4 in both subcellular compartments but did not promote overt changes in its localization.

In nontransformed cells, cell density can induce growth arrest. Hence, we explored whether HDAC4 levels were modulated when growth arrest was induced by density-dependent inhibition. NIH3T3 cells were seeded in 10% FBS, and every 2 d the medium was replaced with fresh 10% FBS. Cytofluorimetric analysis demonstrated that, after 3 d of culture, cells exit the cell cycle and accumulate in G0/G1 phase, similarly to serum-starved cells (Supplemental Figure S1A). Growth arrest was maintained after 7 d of culture (unpublished data). By contrast, HDAC4 protein levels were unchanged during growth arrest induced by density-dependent inhibition (Supplemental Figure S1B).

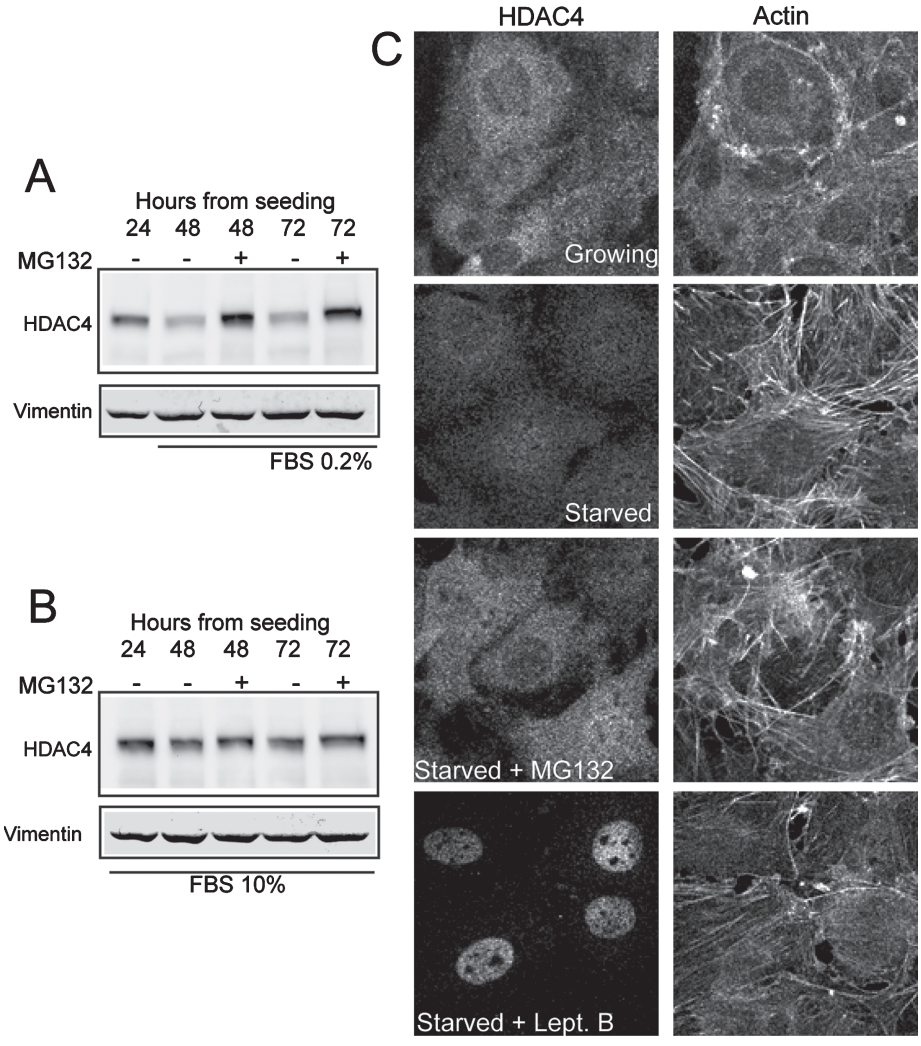

FIGURE 2: Proteasome-dependent degradation of HDAC4 in NIH3T3 cells. (A) NIH3T3 cells were grown for 24 h in 10% FBS (lane 1) and next shifted in low serum (0.2% FBS). When used, MG132 was added to cells 24 h before cell lysis. Cellular lysates were subjected to immunoblot analysis using anti-HDAC4 antibodies. Vimentin was used as loading control. (B) NIH3T3 cells were grown for the indicated times in 10% FBS. When used, MG132 was added 24 h before cell lysis. Cellular lysates were subjected to immunoblot analysis using the specific antibodies. Vimentin was used as loading control. (C) Confocal pictures of growing (24 h after seeding) or serum starved (24 h in 0.2% FBS) NIH3T3 cells. Serum-starved cells were treated with MG132 (2.5 μM) or with leptomycin B (5 ng/ml) for 3 h. Immunofluorescence analysis was performed to visualize HDAC4 subcellular localization. Tetramethylrhodamine-5-(and 6)-isothiocyanate-phalloidin was used to decorate actin filaments.

Conversion of serine 298, sited in PEST1, into an aspartic residue promotes poly-ubiquitination and degradation of HDAC4

PESTs are sequences 10–50 amino acids long containing high densities of Pro, Glu, Ser, and Thr bounded by basic residues that regulate protein stability. Many PEST sequences act as phosphodegrons when they contain phosphorylation sites that mediate interaction with specific E3-ligases. In this manner it is possible to couple substrate degradation to specific environmental signals (Hunter, 2007).

Two PEST sequences (aa 283–331 and aa 559–574) have been described in HDAC4 (Liu *et al.*, 2004). Using the ePESTfind program (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind), we confirmed the existence of the two putative PEST sequences. Only in the PEST1 sequence (aa 283–331), however, can consensus phosphorylation sites (serines 298 and 302) be predicted by in silico analysis. Hence, we decided to investigate in more detail the contribution of PEST1 to HDAC4 stability.

We took advantage of HDAC4 derivatives with serines 298 and 302 mutated to

positively charged aspartate residues to mimic constitutive phosphorylation or mutated to alanine residues to mimic constitutive dephosphorylation. To evaluate the destabilizing effect of the Ser/Asp mutations, the different point-mutated versions of HDAC4 fused to green fluorescent protein GFP were transfected in E1A-transformed cells and, following splitting, cells were treated or not with MG132 in the presence of 10% FBS. We also analyzed the behavior of HDAC4 mutated in all three serines recognized by 14–3-3 proteins (HDAC4-TM), which are important to modulate its subcellular localization.

As shown in Figure 3A, levels of the HDAC4 mutant S298D were clearly augmented by treatment with MG132, whereas other mutants and the wild type (WT) were minimally affected. MG132 could promote an increase in the levels of the S302D mutant, albeit less evident compared to the S298D mutant. In this cell line, mutations of the 14–3-3 binding sites do not overtly influence HDAC4 stability.

Hence, we decided to focus our attention on the S298D mutant. To confirm its destabilizing effect and to exclude differences due to transfection efficiency or to the insertion of the GFP tag, we cotransfected HDAC4 and the point-mutated forms S298D and S298A tagged with a FLAG epitope together with GFP, as a standard for transfection efficiency. After transfection, cells were split and treated or not with MG132. As testified by GFP levels, transfection efficiency was similar under the different conditions (Figure 3B). The S298D mutant was expressed at lower levels compared to the WT and the S298A mutant. MG132 treatment evidently increased the amount of the S298D mutant.

To further investigate the prodegradative effect of the mutation S298D we generated NIH3T3 cells stably expressing HDAC4 WT or its point-mutated derivatives S298A and S298D. Figure 3C shows the levels of the different HDAC4-GFP fusions stably expressed in NIH3T3 cells. Surprisingly, inconsistent with the hypothesized prodegradative effect of the S298D mutation, its level was not strongly reduced, compared to those of the WT. Hence we decided to explore whether the similar expression levels of the WT and of the S298D mutant could be ascribed to diverse amounts of the respective mRNAs, as transcribed in the three cell lines. QRT-PCR analysis demonstrated that, in cells expressing the S298D mutant, the relative mRNA was expressed at much higher levels compared to the WT or the S298A mutant (Figure 3D). It is possible that, in the stable cell lines, the intrinsic instability of the S298D mutant is compensated by a higher amount of its mRNA, compared to the mRNAs encoding for the WT or the S298A mutant.

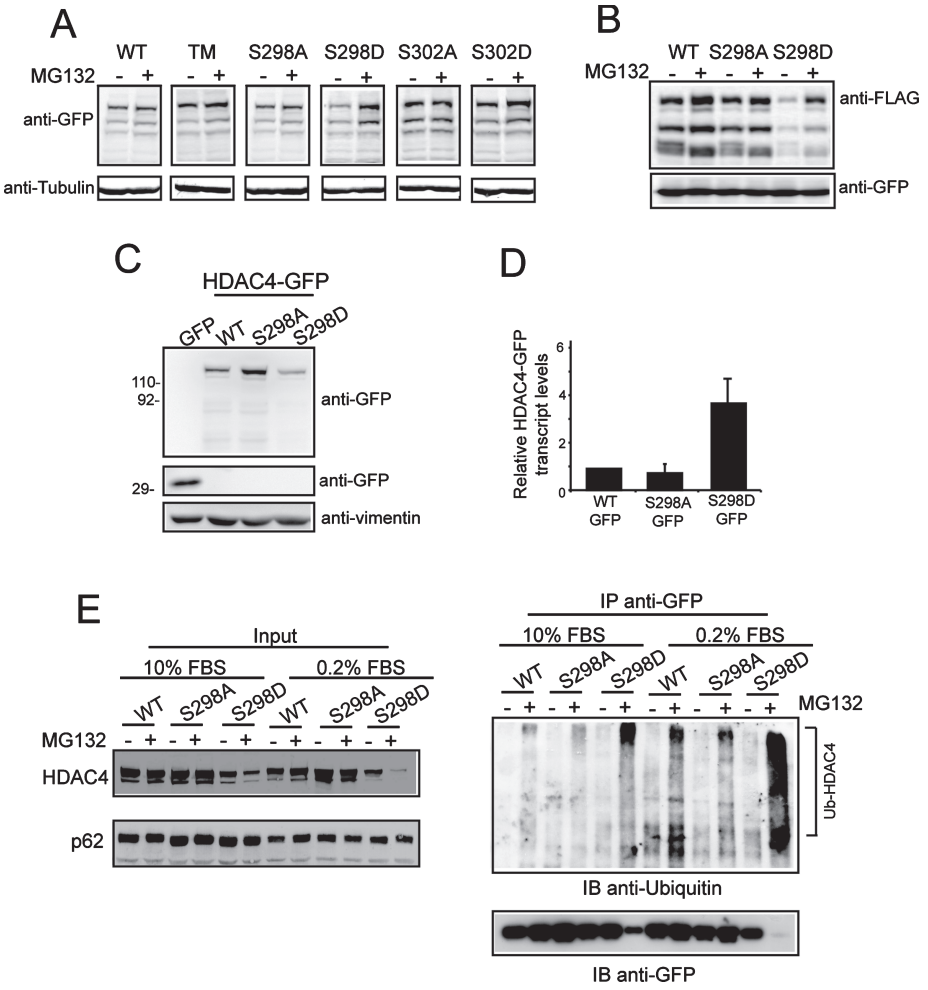

FIGURE 3: Conversion of serine 298 into an aspartic residue, within PEST1 promoted poly-ubiquitination and degradation of HDAC4. (A) Different GFP-fused HDC4 point mutations and the WT form were transfected in IMR90-E1A transformed cells; 24 h later transfected cells were split into two petri dishes and grown for another 24 h. When used MG132 was added 3 h before cell lysis. Cellular lysates were generated and immunoblotted with anti-GFP antibody. Tubulin was used as control. (B) HDAC4 WT and the S298D or A mutants fused to FLAG epitope were cotransfected together with GFP in E1A-transformed cells; 24 h later transfected cells were split into two petri dishes and grown for another 24 h. When used MG132 was added 3 h before cell lysis. Cellular lysates were generated and immunoblotted with anti-FLAG or anti-GFP antibodies. (C) NIH3T3 cells stably expressing GFP or HDCA4 and the S298D/A mutants fused to GFP were lysed, and cellular extracts were subjected to immunoblot analysis using anti-GFP antibodies. Vimentin was used as loading control. (D) Levels of mRNA encoding for HDAC4 or the S298D/A mutants fused to GFP. NIH-3T3 cells were grown in complete medium for 24 h, RNA was extracted, and QRT-PCR analysis was performed to quantify mRNAs of the different fusions. Samples were normalized to β-actin and HPRT (means ± SD, n = 4). (E) NIH3T3 cells stably expressing the different HDAC4-GFP fusions were grown for 24 h in 10% FBS or for an additional 24 h in 0.2% FBS. Cells were lysed as described in *Material and Methods*. HDAC4-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. When used MG132 was added 3 h before cell lysis. Input has been included, and nucleoporin p62 was used as loading control.

In conclusion, in NIH3T3 cells the S298D mutation achieves a prodegradative influence.

To finally prove the effect of the S298D mutation on protein stability we analyzed the poly-ubiquitination of the different HDAC4-GFP fusions. NIH3T3 cells expressing HDAC4 WT, S298D, or S298A mutants were grown in 10% FBS or in low serum and treated with MG132. After cell lysis, immunoprecipitations were performed using an antibody against GFP. Poly-ubiquitinated

HDAC4-GFP fusions were revealed with an antibody against ubiquitin. Figure 3E proves that the S298D mutant was also poly-ubiquitinated when cells were grown in 10% FBS. Its poly-ubiquitination was dramatically increased in serum-starved cells. The WT protein was poly-ubiquitinated almost exclusively after serum withdrawal, whereas, under the same conditions, the mutant S298A was less poly-ubiquitinated compared to the WT (Figure 3E). Curiously, after MG132 treatment, much less protein was immunoprecipitated in the case of the S298D mutant, possibly because of the high levels of poly-ubiquitination and/or aggresome formation (see later in this article). In conclusion, these studies demonstrate that the mutation S298D within PEST1 promotes poly-ubiquitination and degradation of HDAC4.

Effects of proteasomal inhibition on the subcellular localization of HDAC4 and of the 298 mutants

We have previously demonstrated that the S298D mutation impairs HDAC4 nuclear import (Paroni *et al.*, 2008). To better understand the relationships between nuclear import and proteasomal degradation, we analyzed the subcellular localization of the S298D mutant in cells treated with MG132 by time-lapse analysis. Figure 4A illustrates such analysis. Overall, there was an increase in fluorescence intensity after incubation with MG132. In some cells, the S298D mutant remained in the cytoplasm and, subsequently, aggresome-like structures appeared in the cytosol. In other cells (Figure 4A, arrows), the S298D mutant initially accumulated in the nucleus, but over time aggresome-like structures appeared in the cytoplasm and the nuclear fluorescence decreased. Double immunofluorescence analysis performed in cells expressing HA-ubiquitin demonstrated that aggresomes made by the S298D mutant were positive for ubiquitin (Figure 4B).

To gain further insight into the mechanism responsible for the defective nuclear accumulation of the S298D mutant, we analyzed the subcellular localization of the 298 D/A mutants and of the WT protein, after treatment of the cells with MG132, leptomycin B, or a combination of both drugs. Typical examples of the immunofluorescence analysis are shown in Supplemental Figure S2. In untreated cells, the WT protein was localized into the cytosol. Blocking nuclear export elicited its nuclear accumulation, thus demonstrating that HDAC4 undergoes continuous shuttling. Overall, MG132 treatment did not promote an evident nuclear accumulation of HDAC4, but in some cells the nuclear staining was increased (Supplemental Figure S2). The S298A mutant behaves similarly to the WT (Supplemental Figure S2).

The S298D mutant localizes into the cytoplasm and, as previously demonstrated, blocking nuclear export fails to cause its nuclear accumulation (Supplemental Figure S2). When cells were treated with MG132, the mutant chimera accumulated into aggresome-like structures (Supplemental Figure S2), and a weak nuclear fluorescence could be appreciated in several cells. The addition of leptomycin to cells pretreated with MG132 allowed the nuclear accumulation of the S298D mutant, together with the appearance of cytoplasmic aggregates (Supplemental Figure S2).

To better define the pattern of S298D subcellular localization after MG132 and leptomycin treatment, we decided to perform a time-course analysis. Figure 4C demonstrates that MG132 treatment only weakly affected HDAC4 localization, with ~30% of the cells showing both nuclear and cytoplasmic (pan) localization and rare cells with a prominent nuclear staining. A different pattern was observed for the S298D mutant. In this case, a transient nuclear accumulation was evident (~40% of the cells with nuclear accumulation after a 4-h treatment). The nuclear fluorescence declined with time, and simultaneously cytoplasmic aggresomes became evident (Figure 4C), thus confirming the time-lapse analysis. Importantly, the addition of leptomycin to cells pretreated with MG132 efficiently promoted the nuclear accumulation of the S298D mutant, whereas leptomycin alone was ineffective.

These results indicate that the S298D mutant can be imported into the nucleus, where it becomes susceptible to ubiquitin-dependent proteolysis.

GSK3β is required for starvation-induced proteasomal degradation of HDAC4

Having demonstrated that serine 298 plays a pivotal role in the control of HDAC4 stability and because this serine is a predicted GSK3β target, we used knockout cells for this kinase to evaluate its role in the control of HDAC4 degradation.

GSK3$\beta^{-/-}$ and GSK3 $\beta^{+/+}$ fibroblasts were grown for 48 or 72 h in low serum and treated or not with MG132. Figure 5A evidences that, in GSK3β null cells, HDAC4 levels remained constant, also after 72 h of starvation. In contrast, HDAC4 levels decreased when WT cells were grown in low serum, as previously observed for MCF-10A and NIH3T3 cells. This decrease was suppressed by MG132 treatment.

Next we investigated whether GSK3β could directly phosphorylate HDAC4. For this study we generated a new mutant of HDAC4, in which both serines, 298 and 302, were replaced with alanines. The WT HDAC4 and the different mutants fused to GFP were transfected in 293T cells, and immunoprecipitations were performed with an anti-GFP antibody. GFP was also transfected as a negative control. After immunoprecipitation, the different GFP fusions were incubated in a kinase reaction buffer containing [γ-^{32}P]ATP and recombinant GSK3β. Incubation of WT HDAC4 with GSK3β resulted in significant phosphorylation of the deacetylase (Figure 5B). In the absence of the kinase, HDAC4 was not phosphorylated, thus excluding the contribution of eventually coimmunoprecipitated kinases. Phosphorylation of the double mutant (S298A/S302A) was clearly reduced, thus indicating that these serine residues are indeed GSK3β targets. Interestingly, whereas substitution of serine 298 with alanine did not overtly impaired phosphorylation by GSK3β, mutation of serine 302 dramatically affected HDAC4 phosphorylation. This result suggests that serine 302 could serve as a prime phosphorylation site that promotes subsequent phosphorylation of HDAC4 at serine 298 (Jope and Johnson, 2004).

To verify that comparable amounts of the different HDAC4 mutants were immunoprecipitated, an immunoblot was performed with anti-GFP antibody.

HDAC4 and the cellular responses to serum starvation

The regulation of HDAC4 degradation in response to serum starvation prompted us to investigate whether this enzyme could be implicated in transducing growth factor–related responses. Growth factors affect multiple cellular functions, including proliferation, apoptosis, autophagy, and motility. Hence we decided to unveil which among the listed growth-related activities could be modulated by HDAC4. NIH3T3 cells expressing HDAC4 or GFP were used in comparison. Initially we investigated whether the HDAC4-GFP chimera explicated its repressive influence on myocyte-enhancer factor 2 (MEF2) transcription. We analyzed the mRNA levels of the MEF2 target Krüppel-like factor 2 (KLF2), a member of a subclass of the zinc finger family of DNA-binding transcription factors (Kumar *et al.*, 2005; Wang *et al.*, 2010). The QT-PCR results in Figure 6A show that KLF2 mRNA expression was decreased in NIH3T3 cells expressing HDAC4 compared to GFP. Importantly,

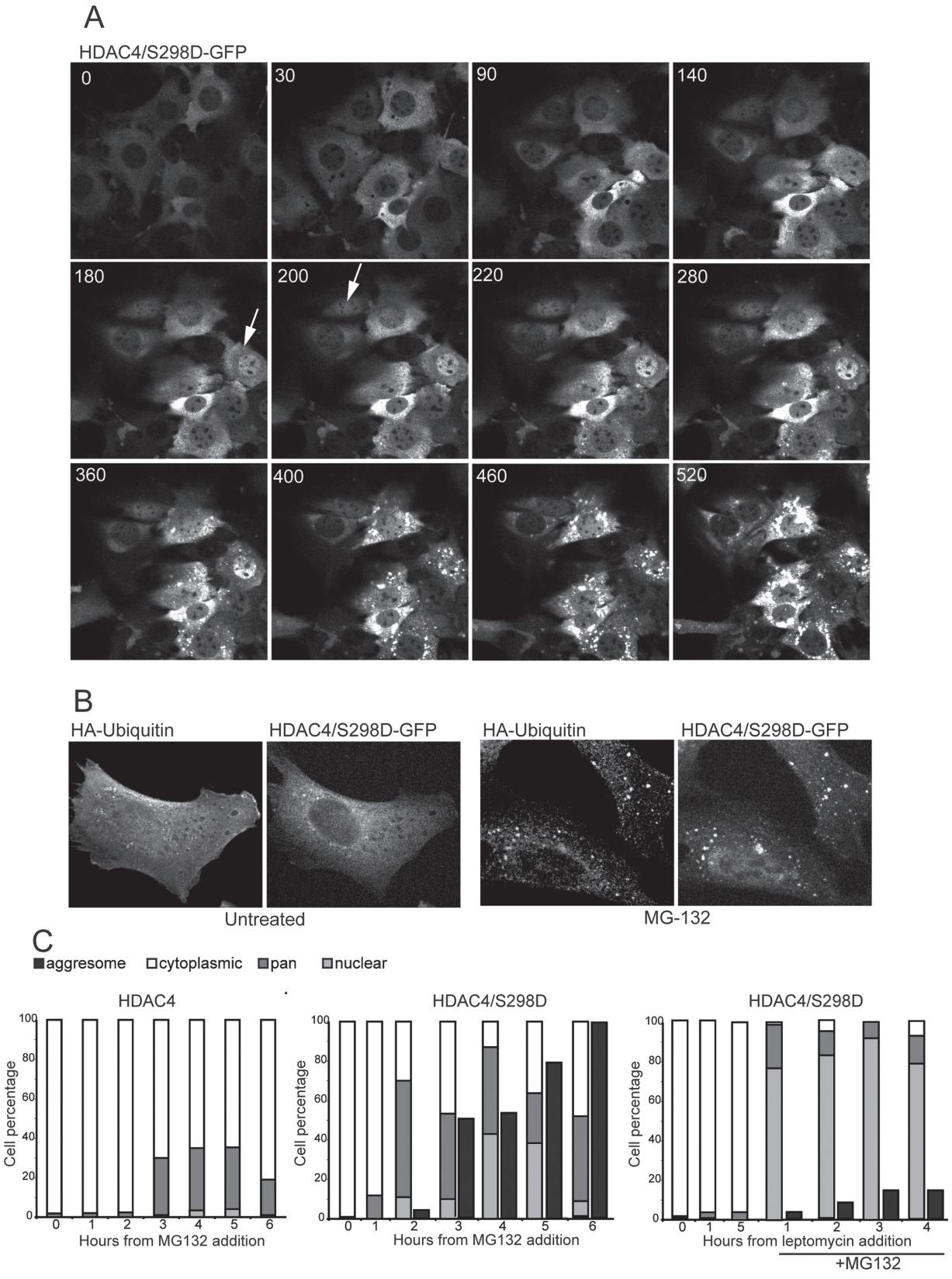

FIGURE 4: Analysis of aggresome formation in NIH3T3 cells expressing the S298D mutant and treated with MG132. (A) Time-lapse analysis of NIH3T3 cell expressing the HDAC4/298D-GFP mutant. Cells were treated with MG132. Frames at selected times (minutes) after addition of MG132 to a representative field are shown. Arrows point to cells that transiently accumulate HDAC4/ S298D-GFP in the nucleus. (B) Confocal microscope images of NIH3T3 cells expressing the HDAC4/S298D-GFP mutant and transfected with HA-tagged ubiquitin. Exponentially growing cells were treated with MG132 for 3 h and then processed for immunofluorescence. (C) Time-course analysis of HDAC4-GFP and HDAC4/S298D-GFP subcellular localization after proteasomal inhibition.

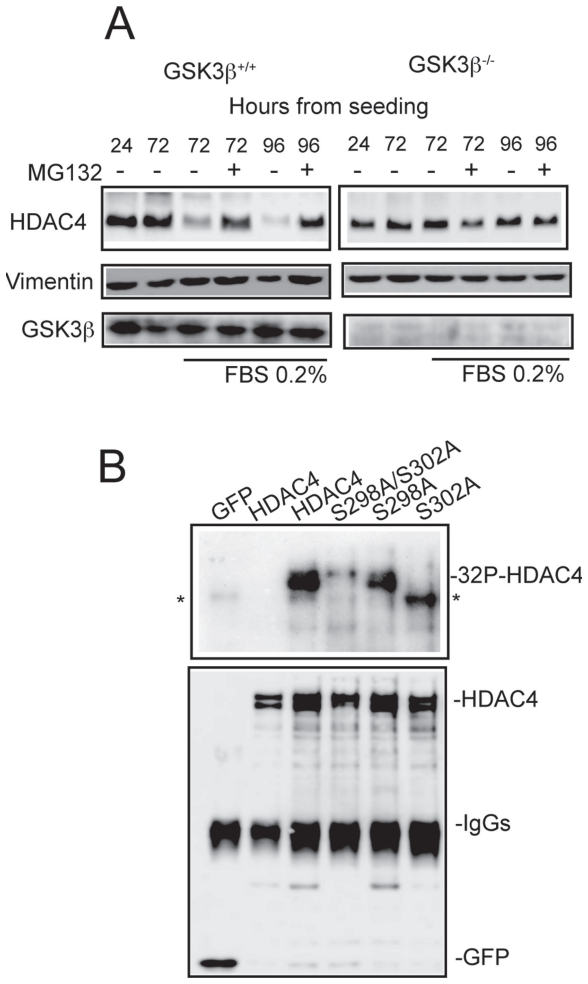

FIGURE 5: GSK3β directly phosphorylates serine 298 and 302 of HDAC4 and modulates its degradation. (A) GSK3β$^{-/-}$ and GSK3 β$^{+/+}$ fibroblasts were grown for 24 h in 10% FBS (lane 1) and next shifted to low serum (0.2% FBS). When used, MG132 was added to cells 24 h before cell lysis. Cellular lysates were subjected to immunoblot analysis using anti-HDAC4 antibodies. Vimentin was used as loading control. (B) Direct phosphorylation of HDAC4 by GSK3β. The top panel shows the result of an in vitro kinase assay after coincubation of recombinant GSK3β with immunoprecipitated HDAC4-GFP, its point mutated versions, or GFP alone, as indicated. In lane 2, WT HDAC4 was incubated in complete kinase assay buffer without the recombinant kinase. The bottom panel shows the loading level of the different immunoprecipitated proteins after immunoblotting with anti-GFP antibody. Asterisks point to a contaminating band.

KLF2 transcript levels were unchanged in cells expressing the S298D mutant, which is defective for repressive activity (Paroni *et al.*, 2008).

Growth factor deprivation can trigger cell death by apoptosis. Hence we evaluated whether cells expressing HDAC4-GFP showed a different susceptibility to apoptosis by low serum conditions. Trypan blue assay and caspase activity (Figure 6, B and C) did not evidence differences in the percentage of apoptosis between serum-starved cells expressing HDAC4-GFP or GFP alone.

In an attempt to adapt to the restrictive environment in terms of nutrients, cells in low serum conditions activate autophagy (Lenk *et al.*, 1999). LC3 (light chain 3) is a specific marker to quantify autophagy in mammalian cells. During autophagy, LC3 undergoes a conversion from the LC3-I isoform to the LC3-II isoform that is specific for autophagosomes (Fontanini *et al.*, 2009). Serum deprivation in NIH3T3 cells induces autophagy (Figure 6D) as evidenced by the appearance of LC3-II. The ectopic expression of HDAC4 did not overtly modulate the appearance of autophagy after serum deprivation.

The main effect of serum starvation in nontransformed cells is the induction of cell-cycle arrest. Hence, we analyzed cell-cycle profiles of cells stably expressing HDAC4-GFP or GFP and grown for different times in low serum. Cytofluorimetric analysis did not reveal changes in the cell-cycle profiles between cells expressing HDAC4-GFP or GFP under the different growth conditions (Figure 6, E–G).

The expression of growth arrest specific (*gas*) genes is up-regulated by serum deprivation. We analyzed the levels of Gas2 protein, which, besides being a marker of G0, is also cleaved by caspase-3 during apoptosis (Brancolini *et al.*, 1995). The immunoblot in Figure 6H confirms that apoptosis was similarly induced in the HDAC4- and GFP-expressing cells, and it also reveals that Gas2 was similarly up-regulated by serum deprivation in the two cell lines.

HDAC4 influences cell motility

Growth factors profoundly affect the architecture of actin cytoskeleton and cell motility. Hence, we finally explored the potential role of HDAC4 in the control of cell motility. Time-lapse microscopy was performed on NIH3T3 cells expressing GFP or HDAC4-GFP, and tracks of individually random migrating cells were analyzed in a computer-aided manner. To evaluate the importance of the MEF2-repressional activity, the motility of cells expressing the S298D mutant was also investigated (Figure 7A). The speed of randomly migrating cells was increased in the presence of HDAC4-GFP compared to GFP alone. Average velocity (arithmetic mean as calculated from the accumulated distance over time) was 0.946 μm/min (SEM = 0.048 n = 78) and 1.468 μm/min (SEM = 0.044; n = 76) for GFP- and HDAC4-GFP–expressing cells, respectively ($p < 0.001$). Interestingly, the random motility of cells expressing the S298D mutant was clearly reduced compared to HDAC4 WT, 1.18 μm/min (SEM = 0.069; n = 54, $p < 0.001$). The difference in migration rate between cells expressing HDAC4 or GFP was even more evident, when data from single-cell analysis were represented as the distance covered by individual cells over time (Figure 7B).

Directional migration is an important component of cell motility that has been shown to be independent from velocity (Pankov *et al.*, 2005). Increased directionality of migration can be quantified by determining the ratio of the shortest, linear distance from the starting point of a time-lapse recording to the end point (D), compared with the total distance traversed by the cell (T) (Pankov *et al.*, 2005). The slight increase in directionality of HDAC4-expressing cells shown in Figure 7C (GFP = 0.33, SEM = 0.025 versus HDAC4 = 0.35, SEM = 0.021) was not statistically significant ($p = 0.38$).

To confirm that HDAC4 regulates random cell motility, we silenced HDAC4-GFP expression. Cells were transfected with small interfering RNA (siRNA) against human HDAC4 or control siRNA. Immunoblot in Figure 7C verified that the siRNA efficiently down-regulated the expression of the HDAC4-GFP transgene. Down-regulation of HDAC4-GFP was coupled to the up-regulation of KLF2 expression, thus confirming that it is an HDAC4 target (Figure 7D).

When we analyzed the speed of randomly migrating cells, overall, a mutual reduction was noted, most likely due to the transfection conditions. Nevertheless, average velocity was significantly reduced in cells transfected with the siRNA against HDAC4 (1.15 μm/min; SEM = 0.056; n = 74) compared to the control (1.35 μm/min; SEM = 0.063; n = 64) ($p < 0.05$).

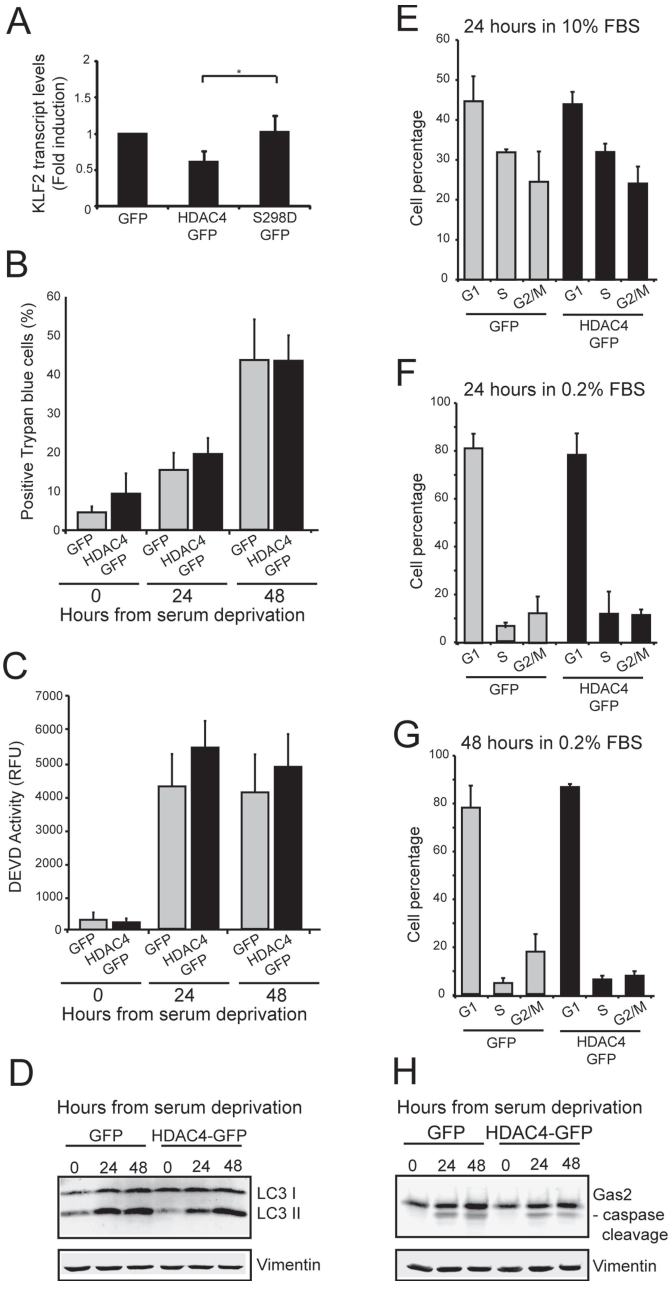

FIGURE 6: Analysis of the cellular responses to low serum conditions in cells expressing HDAC4-GFP. (A) Regulation of KLF2 mRNA expression by HDAC4. RNA was extracted from NIH3T3 cells expressing GFP, HDAC4-GFP, or the point mutant S298D fused to GFP. QRT-PCR analysis was performed to quantify HDAC4 mRNAs. Samples were normalized to GAPDH and HPRT (means ± SD, n = 4). (B) NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were grown for the indicated times in 0.2% FBS. Cell death was scored by trypan blue staining (means ± SD, n = 3). (C) NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were grown for the indicated times in 0.2% FBS. Apoptosis was evaluated by scoring caspase-3/caspase-7 (DEVDase) activities using a fluorogenic assay (means ± SD, n = 3). (D) NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were grown for the indicated times in 0.2% FBS. Equal amounts of cellular lysates were subjected to SDS–PAGE. Immunoblots were performed using an anti-LC3 antibody. Vimentin was used as loading control. (E) Exponentially growing NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were fixed and cell-cycle profiles were assessed after staining with propidium iodide (PI), by fluorescence-activated cell sorting (FACS) analysis (means ± SD, n = 3). (F) After 24 h of serum starvation, NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were fixed, and cell-cycle profiles were assessed after staining with PI, by FACS analysis (means ± SD, n = 3). (G) After 48 h of serum starvation, NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were fixed, and cell-cycle profiles were assessed after PI staining, by FACS analysis (means ± SD, n = 3). (H) NIH3T3 fibroblasts expressing HDAC4-GFP or GFP were grown for the indicated times in 0.2% FBS. Equal amounts of cell lysates were subjected to SDS–PAGE. Immunoblots were performed using an anti-Gas2 antibody. Vimentin was used as loading control.

Finally, we analyzed whether HDAC4 could influence random motility in serum-starved cells. Average velocity was dramatically reduced after 24 h of serum starvation (Supplemental Figure S3) in GFP-expressing cells (0.64 μm/min, SEM = 0.046, n = 54). A similar reduction was observed in the case of WT-expressing cells (0.67 μm/min, SEM = 0.054, n = 43) as well as in S298A-expressing cells (0.69 μm/min, SEM = 0.053, n = 60). These results demonstrate that HDAC4 is not sufficient to counteract the decline in random cell motility, operated by serum withdrawal. Moreover, they indicate that this pathway governing random cell motility is subjected to multiple levels of regulation.

DISCUSSION

HDAC4 degradation

With this work we have added another piece to the complexity of HDAC4 and, more in general, of class IIa HDAC regulation. We demonstrate that serum and growth factors subordinate HDAC4 activity through the control of its ubiquitination and degradation. Serum removal does not influence HDAC4 shuttling, which was similarly observed in growing and starved cells; instead, it elicits HDAC4 poly-ubiquitination and degradation.

Our data reconcile previously published controversial results on proteasome-mediated degradation of HDAC4 (Li *et al.*, 2004; Potthoff *et al.*, 2007). In fact, we have demonstrated that HDAC4 poly-ubiquitination and degradation is a signal-regulated process. It is important to note that HDAC4 degradation could take place not only after serum and/or growth factor removal but also in the presence of serum, when specific signals are provided (Potthoff *et al.*, 2007; Ishikawa *et al.*, 2010).

We have identified specific serines within the PEST1 domain of HDAC4 as important determinants of HDAC4 stability. In particular, mutation of serine 298 into aspartate, which mimics constitutive phosphorylation, causes HDAC4 instability. This mutated HDAC4 is constitutively poly-ubiquitinated and accumulates into aggresomes after proteasomal degradation is suppressed. This behavior is also indicative of an altered protein folding (Rodriguez-Gonzalez *et al.*, 2008). It will be interesting to investigate whether phosphorylation of serine 298 might influence HDAC4 folding and whether this change might represent the switch that allows its poly-ubiquitination.

We previously showed that the S298D mutation affects HDAC4 nuclear import (Paroni *et al.*, 2008). We now demonstrate that when the proteasome is inhibited and leptomycin B is added, this mutant can accumulate into the nucleus. This result indicates that HDAC4 degradation takes place into the nucleus. It would be interesting to investigate whether sumoylation of HDAC4, which occurs during nuclear import, could influence its stability (Kirsh *et al.*, 2002).

The amino acid sequence comprising serines 298 and 302 is highly conserved among class IIa HDACs with the exception of HDAC7. Hence, it is possible that similar mechanisms also operate on HDAC5 and 9 to regulate their stability. In contrast, HDAC7,

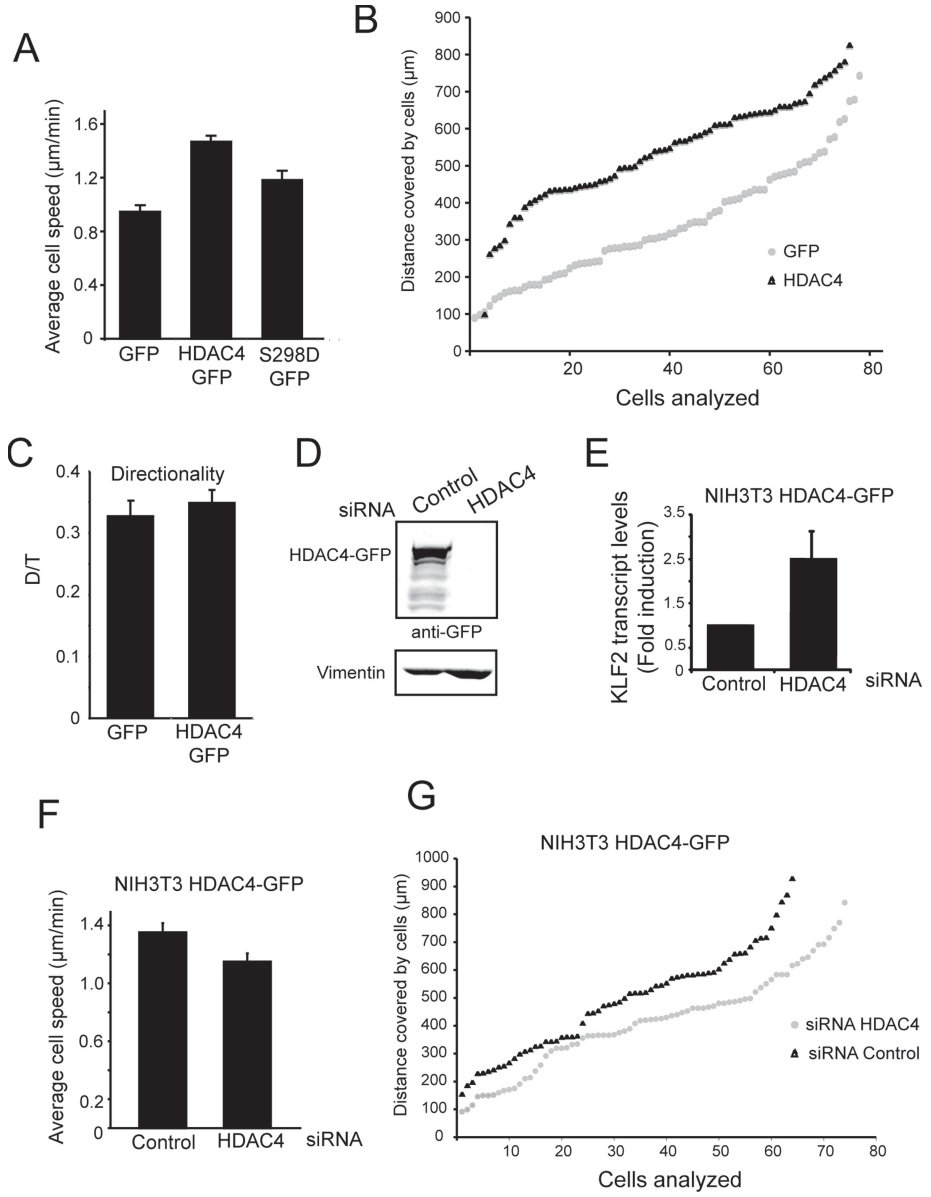

FIGURE 7: HDAC4 influences cell motility. (A) After 24 h from seeding, NIH3T3 cells expressing HDC4-GFP or GFP were subjected to time-lapse analysis for 6 h. Results represent the average migration rate from at least 54 cells from four independent experiments. Cell movements were quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA). (B) Total distance covered by cells expressing GFP or HDAC4-GFP during the time of analysis, as analyzed by time lapse microscopy. Each position along the *x* axis represents a single cell. (C) Quantification of the persistence of migratory directionality. D/T ratios represent the ratio of the direct distance from start to end point (D) divided by the total track distance (T). (D) NIH3T3 cells expressing HDAC4-GFP were transfected with an siRNA against human HDAC4 or a control siRNA. Cellular lysates were generated and immunoblotted with anti-GFP antibody. Vimentin was used as control. (E) NIH3T3 cells expressing HDAC4-GFP were transfected with an siRNA against human HDAC4 or a control siRNA. RNA was extracted, and QRT-PCR analysis was performed to quantify KLF2 mRNA. Samples were normalized to β-actin, and HPRT (means ± SD, n = 4). (F) NIH3T3 cells expressing HDAC4-GFP were transfected with an siRNA against human HDAC4 or a control siRNA; 36 h later, cells expressing HDC4-GFP or GFP were subjected to time-lapse analysis for 6 h. Results represent the average migration rate from at least 64 cells from three independent experiments. (G) Total distances covered by cells expressing HDAC4-GFP and transfected with control or HDAC4 siRNAs are as indicated. After transfection, cells were subjected to time-lapse analysis. Each position along the *x* axis represents a single cell.

which lacks the corresponding serine 298 of HDAC4, could be subjected to different controls (Li *et al.*, 2004). Serines 298 and 302 are placed within consensus sites for GSK3β kinase. GSK3β has been found to regulate the proteolysis of a large number of proteins, in a growth factor–dependent fashion (Xu *et al.*, 2009). We have shown that cells lacking GSK3β are unable to degrade HDAC4 after serum starvation and that GSK3β can phosphorylate HDAC4 in vitro.

GSK3β is unique in requiring a priming phosphate at *n*+4 to phosphorylate many of its substrates (Frame and Cohen, 2001). The double mutation of serines 298/302 into alanines, but also the sole mutation of serine 302, abolishes HDAC4 phosphorylation by GSK3β. These results suggest that serine 302 could act as a priming phosphorylation site. Further studies will be necessary to characterize in detail the pattern of PEST1 phosphorylation and to clearly establish the priming activity of serine 302. Interestingly, in silico analysis highlights that serine 302 could be a substrate both of extracellular-regulated kinase and GSK3β. Hence, control of HDAC4 degradation in cells could undergo a more complex regulation (Zhou *et al.*, 2000).

In summary, proteasomal degradation is a more radical strategy to switch off HDAC4 activity, including "cytosolic functions" (Chen and Cepko, 2009), whereas regulation of nuclear cytosolic shuttling could be more suited for the control of the HDAC4 "nuclear functions."

HDAC4 and cell motility

We have observed that several cellular activities under the control of serum, such as cell-cycle progression, growth arrest, apoptosis, or autophagy, were unaffected by HDAC4. Instead we have noted that random cell motility is augmented in cells expressing HDAC4.

Previous studies have revealed possible roles of class IIa HDACs in the regulation of cell migration and motility. HDAC7 dosage has important implications on endothelial cell motility and migration. Either the silencing of HDAC7 (Mottet *et al.*, 2007) or the overexpression of signal-resistant HDAC7 (mutated in the serine binding sites for 14–3-3) impairs cell motility (Wang *et al.*, 2008). In endothelial cells, siRNA directed against HDAC5 sustained the migration, whereas an opposite effect was exerted by siRNA against HDAC7 and HDAC9. In this model, overexpression of HDAC5 suppressed angiogenesis in an MEF2-independent manner (Urbich *et al.*, 2009). This study confirms a previous report of a negative role of HDAC5 in the control of endothelial cell migration in response to vascular endothelial growth factor (Ha *et al.*, 2008). Although a role of class IIa HDAC in the control of cell motility is emerging, there are some discrepancies. Divergences could be

ascribed to the specific roles of the different HDACs, to the diverse cellular/environmental context, or to the assays used to score cell motility.

Cells can migrate randomly (random cell motility) or can maintain a direction (directionally persistent cell migration) even with no external chemotactic signals, using intrinsic cell migration properties (Pankov *et al.*, 2005; Petrie *et al.*, 2009). In our assay, we have noted that HDAC4 increases cell speed and random cell motility but not directionally persistent migration.

How could HDAC4 modulate cell motility? Genes involved in orchestrating actin cytoskeleton, the expression of which is modulated by HDAC4, are strong candidates to explain the motility effect of the deacetylase. We have shown that, in our cellular model, HDAC4 controls the expression of the MEF2-target gene KLF2. Recent studies have demonstrated that in endothelial cells KLF2 can manage the organization of actin cytoskeleton in response to shear stress (Boon *et al.*, 2010). In cells overexpressing KLF2, focal adhesion kinase was dephosphorylated, and activation of the small GTPase RhoA was amplified (Boon *et al.*, 2010). HDAC4 could antagonize this effect, thus promoting random cell motility.

In addition to the nuclear related functions, we cannot exclude a contribution of the cytosolic HDAC4, which can associate with cardiac myofilaments and modulate the acetylation of Z-disk–associated proteins (Gupta *et al.*, 2008). Moreover, HDAC4 was shown to interact with the actin binding protein α-actinin 4 (Chakraborty *et al.*, 2006; Paroni *et al.*, 2008).

In conclusion, we propose that HDAC4 could modulate the responsiveness to specific mitogenic signals. In our hypothesis, degradation of HDAC4, as triggered by growth factor removal, could be part of the integrated cellular response to fine-tuning its motility to mutated environmental conditions.

MATERIALS AND METHODS

Cell culture

MCF-10A cells were maintained in Ham's F12/DMEM 1:1 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), insulin (0.01 mg/ml), hydrocortisone (500 ng/ml), epithelial growth factor (20 ng/ml), and cholera toxin (100 ng/ml). $MEF^{GSK3b-/-}$ $MEF^{GSK\beta+/+}$, IMR90-E1A, MCF-7, 293T, and NIH3T3 cells were maintained in DMEM supplemented with 10% FBS plus penicillin/streptomycin and L-glutamine. The proteasome inhibitor MG132 (Calbiochem, Gibbstown, NJ) was used at 2.5 µM when added for 24 h and at 5 µM when added for 3 or 6 h. The CRM1 inhibitor, leptomycin-B (LC Laboratories, Woburn, MA), was used at 5 ng/ml.

Plasmid construction, transfection, and retroviral infection

pFLAG-CMV5 and pEGFPN1 constructs expressing HDAC4 and its mutants were previously described (Paroni *et al.*, 2008). The double mutant S298A and S302A was generated by in vitro mutagenesis using the Gene-Taylor kit (Invitrogen, Carlsbad, CA) and pEGFPN1-*HDAC4-S298A* as template. Oligonucleotide sequences are available upon request. Plasmid transfections in IMR90-E1A and 293T cells were performed with the calcium phosphate method. NIH3T3 cells expressing the different transgenes were generated by retroviral infection after cloning of *GFP* or GFP-tagged *HDAC4* WT, *HDAC4 S298A*, or *HDAC4 S298D* mutants into pWZL-Hygro retroviral vector, as described previously (Fontanini *et al.* 2009).

Immunoblotting and immunoprecipitation

Proteins obtained after an SDS denaturing lysis and sonication were transferred to a 0.2-µm-pore-sized nitrocellulose membrane and incubated with the following antibodies: anti-HDAC4, anti-GFP, anti-tubulin (Paroni *et al.*, 2004), anti-FLAG-M2 (Sigma Aldrich, St. Louis, MO), anti-vimentin, anti-Gas2 (Brancolini *et al.*, 1995), anti-ubiquitin (Covance, Princeton, NJ), anti-GSK3 (Invitrogen, Carlbad, CA), anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then rinsed three times with Blotto/Tween 20 and incubated with the relative secondary antibody (Euroclone, Milan, Italy) for 1 h at room temperature. Blots were then washed three times in Blotto/Tween 20, rinsed in phosphate-buffered saline, and developed with Super Signal West Pico, as recommended by the vendor (Pierce, Rockford, IL).

Immunoprecipitations were performed as previously described (Fontanini *et al.*, 2005). Briefly, cells were collected directly from culture dishes with a rubber scraper into RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.2% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate), supplemented with 50 mM iodoacetamide, 1 µM isopeptidase inhibitor G5 (Fontanini *et al.*, 2009), 1 µM MG132, and protease inhibitors. Lysates were incubated for 6 h with the antibody against HDAC4 or 3 h with the antibody against GFP. After 1 h of incubation with protein A beads (GE, Chalfont St. Giles, UK), washes were performed with RIPA buffer and finally three times with 50 mM Tris-HCl, pH 8. Samples were resolved by SDS–PAGE and analyzed by immunoblot.

In vitro phosphorylation

HDAC4 WT and the different mutants fused to GFP or GFP alone were transfected in 293T cells and immunoprecipitated. After several washes, the different GFP fusions were incubated in the kinase reaction buffer (5 mM 3-(N-morpholino)-propanesulfonic acid [MOPS], pH 7.2, 2.5 mM β-glycerophosphate, 1 mM ethylene glycol tetraacetic acid, 0.4 mM EDTA, 2 mM $MgCl_2$, 50 µM dithiothreitol) containing 10 µM ATP and 300 nM [γ-^{32}P]ATP. Recombinant GST-GSK3β (100 ng; Cell Signaling Technology, Danvers, MA) was added and beads were incubated for 30 min at 30°C. After several washes, samples were resolved by SDS–PAGE, and proteins were transferred to a nitrocellulose membrane. Film exposure to the membrane was used to reveal the amount of phosphorylated proteins, and subsequent immunoblotting of the membrane was used to verify the amount of immunoprecipitated proteins.

RNA extraction and QRT-PCR

Cells were harvested and RNA was obtained using TRIZOL (Invitrogen). RNA integrity was checked by running a formaldehyde-agarose gel. Total RNA (2.5 µg) was used for retrotranscription. QRT-PCR was performed using the Bio-Rad iQ5 or the Bio-Rad CFX96 and SYBR Green technology. To analyze data obtained from QRT-PCR experiments, we used the delta-delta Ct method. In the case of the MCF-10A cells, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was selected for normalization. In the case of the NIH3T3 cells, the geometric mean of the threshold cycles for HPRT (hypoxanthine phosphoribosyltransferase) and β-actin was selected as the normalization factor. All reactions were done in triplicate. All primer sequences used in this article are available upon request.

Cell-cycle analysis

Cells were detached by trypsin and fixed in 70% ethanol. After some washing, cells were resuspended in PBS supplemented with 1% Triton X-100 and RNAse-A at 100 µg/ml and were incubated for 30 min at 37°C. DNA staining was performed by incubating cells with propidium iodide at 50 µg/ml for 45 min at room temperature. Cells were then passed through a flow cytometer equipped with CellQuest software by using a 488-nm argon ion laser (FACScan; BD Biosciences, Franklin Lakes, NJ). A minimum of 10,000 events per

sample were analyzed. Data analysis was performed by MODFIT software (BD Bioscience, Franklin Lakes, NJ).

Random motility measurements

Random motility was assayed by time-lapse video microscopy analysis of low-density cultured cells. Cells were analyzed at 24 h from plating or at 24 h from starvation. In each experiment, 10× phase contrast time-lapse images were acquired every 15 min during a 6-h period for at least two representative fields for experimental condition. Time-lapse images were analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA). After the center of each cell was defined, its movement was scored throughout the time sequence. The average cell velocity was obtained (μm/min), and the directionality was measured as the ratio between the direct distance from the start to the end point (D) divided by the total track distance (T). Results are pooled from eight independent experiments; error bars indicate SEM based on $N \geq 54$.

ACKNOWLEDGMENTS

We thank Davide Orlando for helping in some experiments and Francesca Demarchi, Laboratorio Nazionale Consorzio Interuniversitario per le Biotecnologie (LNCIB) Trieste, for providing reagents and cell lines. This work was supported by AIRC (Associazione Italiana Ricerca sul Cancro), MIUR (Ministero dell'Istruzione, Università e Ricerca), and Regione Friuli-Venezia Giulia (AITT-LR25/07) to C.B.

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