

Class IIa histone deacetylases: conducting development and differentiation

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ABSTRACT The emergence of specialized cell types and their organisation into organs and tissues involve the temporal modulation of many genes that are essential for coordinating the correct timing of instructive signals. These transcriptional changes are orchestrated with a precision that reminds that of a classical symphony. Extracellular signals are transmitted to key «integrators», which then orchestrate activation or repression of specific genes. In the last decade, class IIa HDACs have emerged as crucial regulators in various developmental and differentiation processes. This review focuses on the latest studies that have provided new insights into the biological functions of class IIa HDACs and discusses important aspects of their regulation. Elucidating cellular and molecular mechanisms by which functions of class IIa HDACs are modulated could potentially lead to new therapeutic opportunities for various diseases.

KEY WORDS: *DAC, nucleocytoplasmic shuttling, acetylation, chromatin, MEF2*

The classification of the eighteen human Histone Deacetylases (HDACs) identified to date relies on biochemical, structural and phylogenetic criteria. Based on their dependence on specific co-factors, a first division separates group I HDACs, which are zinc-dependent amidohydrolases, from group II enzymes (also known as class III HDACs or SIRT6s), which rely on nicotinamide adenine dinucleotide (NAD) for their catalytic activity (Haigis and Guarente, 2006). Group I HDACs are further subdivided into classes I, II and IV, based on their similarity to yeast proteins (Yang and Seto, 2008). While class I HDACs (HDAC1,-2,-3 and -8) are related to the yeast transcriptional regulator Rpd3 (Rundlett *et al.*, 1996; Taunton *et al.*, 1996), class II enzymes (HDAC4,-5,-6,-7,-9,-10) show more similarity to yHDA1, a Rpd3-paralog (Fischer *et al.*, 2002; Fischle *et al.*, 2001; Fischle *et al.*, 1999; Grozinger *et al.*, 1999; Guardiola and Yao, 2002; Kao *et al.*, 2000; Miska *et al.*, 1999; Petrie *et al.*, 2003; Tong *et al.*, 2002; Verdell and Khochbin, 1999; Wang *et al.*, 1999; Zhou *et al.*, 2001; Zhou *et al.*, 2000a). Careful phylogenetic analyses have singled HDAC11 out from other group I HDACs and led to the idea that it might be the defining member of a separate fourth class (Gregoretti *et al.*, 2004).

Based on their structure, class II HDACs are further divided into two subclasses: class IIa (HDAC4, -5, -7 and -9) and class IIb (HDAC6 and -10) (Verdin *et al.*, 2003). Class IIa HDACs are the subject of this review and show a very specific structural organi-

zation, that includes two distinct domains. The catalytic HDAC domain lies within the C-terminal half of the protein and corresponds to the HDA1-like sequences. The N-terminal part of class IIa HDACs is the hallmark of the subfamily and contains specific conserved amino acid motifs that are specialized for binding an array of proteins, such as DNA-binding transcription factors, transcriptional corepressors and chaperone proteins (Martin *et al.*, 2007). This adaptor domain of class IIa HDACs also contains several motifs specifically involved in their regulation. A canonical nuclear localization signal is found in the N-terminus of all class IIa members and controls their subcellular localization (McKinsey *et al.*, 2000a; McKinsey *et al.*, 2001; Wang and Yang, 2001). In addition, several conserved residues are targeted by various post-translational modifications, such as ubiquitination (Li *et al.*, 2004), sumoylation (Kirsh *et al.*, 2002; Petrie *et al.*, 2003), phosphorylation (Grozinger and Schreiber, 2000; Wang *et al.*, 2000) and proteolytic cleavage (Bakin and Jung, 2004; Li *et al.*, 2004; Paroni *et al.*, 2004; Scott *et al.*, 2008).

According to the current view, class IIa HDACs are thought to act as transcriptional co-repressors by deacetylating nucleoso-

Abbreviations used in this paper: CAMK, Ca²⁺/calmodulin-dependent kinase; HDAC, histone deacetylase; MEF, myocyte enhancer factor; NAD, nicotinamide adenine dinucleotide; PKD, protein kinase D.

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mal histones in the vicinity of target promoters. Since class IIa HDACs do not bind to DNA, their repressive activity relies on interactions with sequence-specific DNA binding proteins, which thus dictate the targeting specificity of class IIa HDACs. The canonical example of this model is illustrated by the interaction between class IIa HDACs and myocyte enhancer factor 2 (MEF2) transcription factors (Martin *et al.*, 2007). However, this simplistic model is challenged by a series of experimental evidence. It is notorious that compared to class I or class IIb, class IIa HDACs are very inefficient histone deacetylases (Fischle *et al.*, 2002; Fischle *et al.*, 1999; Hassig *et al.*, 1998; Hu *et al.*, 2000). An explanation for this intrinsic weak HDAC activity came from a recent study showing that, in place of a catalytic tyrosine residue that is strictly conserved in other group I enzymes, class IIa HDACs have an histidine that compromises their activity towards acetyl-lysines (Lahm, 2007). Very importantly, these findings raise the intriguing possibility that class IIa HDACs might in fact not target acetylated proteins, but still-to-be-discovered substrates. In addition, class IIa HDACs can repress transcription independently of their C-terminal catalytic domain, which suggests that at least part of their repressive properties does not rely on deacetylation of proximal histones (Kao *et al.*, 2000; Lemerrier *et al.*, 2000; Sparrow *et al.*, 1999; Wang *et al.*, 1999; Zhou *et al.*, 2000a). Indeed, the isolated N-terminal region of class IIa HDACs can repress transcription by recruiting co-repressors, such as HP1 or CtBP (Zhang *et al.*, 2002; Zhang *et al.*, 2001a; Zhang *et al.*, 2001b). Finally, class IIa HDACs can also modulate the transcriptional activity of their partners by regulating specific post-transcriptional modifications, such as sumoylation (Gregoire *et al.*, 2006; Gregoire and Yang, 2005; Zhao *et al.*, 2005) or ubiquitination (Jeon *et al.*, 2006; Jin *et al.*, 2004).

In addition to their similar structural organization, class IIa HDACs also share the same overall mechanism of regulation. Regulation of class IIa members relies on their ability to shuttle between the nucleus and the cytoplasm, in response to specific extracellular signals (Martin *et al.*, 2007). When in the nucleus, class IIa HDACs can partner with transcription factors and co-repressors to inhibit transcription. In contrast, cytoplasmic accumulation of class IIa HDACs makes them unable to impact on transcription as it sequesters them away from histones and renders them enzymatically inactive as histone deacetylases (Fischle *et al.*, 2001; Fischle *et al.*, 2002). Multiple lines of evidence suggest that class IIa HDAC subcellular localization relies on the phosphorylation of several conserved serine residues located in their adaptor domain. When phosphorylated, these residues are targeted for binding by 14-3-3 proteins, which promote CRM1-dependent nuclear export and/or cytoplasmic sequestration of class IIa HDACs (Grozinger and Schreiber, 2000; Kao *et al.*, 2001; Liu *et al.*, 2005; Wang *et al.*, 2000). In contrast, mutation of the phosphorylatable residues into alanine generates shuttling deficient class IIa HDACs and abolishes the signal-responsiveness of their target genes (Dequiedt *et al.*, 2003; Ha *et al.*, 2008; McKinsey *et al.*, 2000a; Miska *et al.*, 2001; Wang *et al.*, 2008; Zhang *et al.*, 2002; Zhang *et al.*, 2001b). As described for several of their target proteins, 14-3-3 are thought to directly control the subcellular localization of class IIa HDACs by modulating the function of their NES and NLS (Grozinger and Schreiber, 2000; McKinsey *et al.*, 2000a; Muslin and Xing, 2000). However, a series of important observations have been over-

looked that are not compatible with this model. First, despite having intact 14-3-3 binding sites, HDAC5 mutants harboring inactivating mutations in their NES remain nuclear. But most importantly, the constitutive nuclear localization of these NES mutants does not increase their repressive activity. This indicates that release from their targeting DNA-binding partners, rather than exit from the nucleus is the crucial step in the relief of class IIa HDAC transcriptional repression by phosphorylation (Lu *et al.*, 2000a; McKinsey *et al.*, 2000a). In this context, 14-3-3-dependent nuclear export of class IIa HDACs may only serve as a supporting mechanism that ensures maximal activation of their target genes (McKinsey *et al.*, 2000a). In addition, some data suggest that association with 14-3-3 could be dispensable for CRM1-mediated nuclear export of class IIa HDACs (Gao *et al.*, 2006). Full comprehension of the complex mechanisms governing class IIa HDAC subcellular localisation and activity undoubtedly requires further investigation in order to solve these important issues.

As described above, inhibition of class IIa HDAC phosphorylation by serine-to-alanine mutation of the 14-3-3 consensus sites, leads to nuclear accumulation and increased repressive activity. This suggests that modulation of class IIa HDAC phosphorylation would provide the unique opportunity to control their biological functions. Indeed, activation of class IIa HDAC target genes can be achieved by overexpression of protein kinases or inhibition of protein phosphatase, each leading to hyperphosphorylation and cytoplasmic accumulation of class IIa HDACs. A great deal of effort has thus been invested in identifying the protein kinases and phosphatases targeting class IIa HDAC 14-3-3 motifs. To date, four families of serine/threonine kinases and two families of phosphatases have been implicated in this mechanism. Historically, members of the Ca²⁺/calmodulin-dependent kinase family (CaMK), specifically CaMKI and IV, were the first kinases shown to promote 14-3-3 binding and nuclear export of class IIa HDACs. Functional relevance of these findings was provided in various biological contexts in which activation of CaMK members correlates with activation of class IIa HDAC targets genes (Backs *et al.*, 2006; Bossuyt *et al.*, 2008; Chawla *et al.*, 2003; Davis *et al.*, 2003; Grozinger and Schreiber, 2000; Kao *et al.*, 2001; Karamboulas *et al.*, 2006b; Linseman *et al.*, 2003; Lu *et al.*, 2000b; McKinsey *et al.*, 2000b). However, these studies mainly rely on overexpression or pharmacological inhibition of CaMK and remain to be validated by more definite loss-of-function approaches. More recently, protein kinase D (PKD), a downstream effector of PKC was shown to phosphorylate the 14-3-3 binding sites of class IIa HDACs and neutralize their repressive activity (Dequiedt *et al.*, 2005; Matthews *et al.*, 2006; Parra *et al.*, 2005; Vega *et al.*, 2004a). Convincing experimental evidence have demonstrated that PKD activation is necessary for inactivation of class IIa HDACs during T-cell apoptosis (Dequiedt *et al.*, 2005; Parra *et al.*, 2005), cardiac hypertrophy (Bossuyt *et al.*, 2008; Vega *et al.*, 2004a), B-cell receptor signaling (Matthews *et al.*, 2006), skeletal and cardiac muscle remodeling (Fielitz *et al.*, 2008; Kim *et al.*, 2008a) and angiogenesis (Ha *et al.*, 2008; Wang *et al.*, 2008). Mice with reduced CREB activity develop muscular dystrophy because of increased expression of salt-inducible kinase 1 (SIK1). Interestingly these mice also exhibit hyperphosphorylation of HDAC5, suggesting that SIK1 could be a class IIa HDAC kinase. Indeed loss- and gain-of-function studies demonstrated that SIK1 phosphorylates the 14-3-3 binding sites of HDAC5, revealing an important CREB-

SIK1-HDAC5-MEF2 axis in the regulation of the myogenic program (Berdeaux *et al.*, 2007; Takemori *et al.*, 2009). However, whether this pathway is also conserved in other MEF2-regulated programs remains totally unexplored. In addition to the signal-regulated protein kinases described above, two members of the microtubule affinity-regulating kinase (MARK)/Par-1 family, which are usually considered as constitutively active kinases (Lizcano *et al.*, 2004), were shown to directly phosphorylate and promote nuclear export of class IIa HDACs (Chang *et al.*, 2005; Dequiedt *et al.*, 2006). Unfortunately, despite strong biochemical data these studies lack supporting evidence to demonstrate the biological relevance of their findings.

Considering the reversible nature of protein phosphorylation, protein phosphatases should be as important as protein kinases in the regulation of class IIa HDACs. Indeed, Parra *et al.* recently reported dephosphorylation of HDAC7 by the myosin phosphatase complex, which contains PP1 β and myosin phosphatase targeting subunit 1 (MYPT1) (Parra *et al.*, 2007). In addition, results from our laboratory and others have revealed that another cellular phosphatase, PP2A, stably associates with and constitutively dephosphorylates class IIa HDACs *in vivo* (Martin *et al.*, 2008; Paroni *et al.*, 2008). Accordingly, PP2A participates in the regulation of class IIa HDAC subcellular localization and directly impacts on their biological functions (Illi *et al.*, 2008; Martin *et al.*, 2008; Paroni *et al.*, 2008; Sucharov *et al.*, 2006). The sequential and/or coordinated actions of these multiple protein kinases and phosphatases would constitute a tightly regulated mechanism allowing appropriate, rapid, and reversible expression of class IIa HDAC target genes in response to specific developmental signals.

In addition to phosphorylation of their 14-3-3 binding sites, class IIa HDACs are subject to additional posttranslational modifications: phosphorylation/dephosphorylation at serine residues distinct from the 14-3-3 binding sites (Deng *et al.*, 2005; Paroni *et al.*, 2008; Zhou *et al.*, 2000b), sumoylation (Kirsh *et al.*, 2002; Petrie *et al.*, 2003; Tatham *et al.*, 2001), ubiquitination (Hook *et al.*, 2002) and caspase cleavage (Li *et al.*, 2004; Paroni *et al.*, 2004; Scott *et al.*, 2008). For more details on how these modifications impact on localization, stability, repressive or catalytic activity of specific members of class IIa HDACs, we respectfully refer the reader to recently published reviews (Martin *et al.*, 2007; Yang and Seto, 2008).

Recently, two additional mechanisms of class IIa HDACs regulation have been documented. First, HDAC4 was shown to oligomerize with HDAC5, and to a lesser extent with HDAC9. Interestingly, amongst the class IIa HDACs, HDAC4 is uniquely sensitive to CaMKII-mediated phosphorylation and nuclear export (Backs *et al.*, 2006). Hetero-oligomerization with HDAC4 brings HDAC5 in close proximity of CaMKII, allowing its phosphorylation by the kinase and promoting its nuclear export (Backs *et al.*, 2008). Whether this transphosphorylation mechanism also exists for other class IIa HDACs and as-yet-undefined specific kinases remains unknown. In addition, two studies recently reported that expression of HDAC4 is regulated at the post-transcriptional level by microRNAs (miRNA). During skeletal muscle differentiation, miR-1 promotes myogenesis by targeting HDAC4

(Chen *et al.*, 2006). Similarly, miR140, a cartilage specific miRNA, can bind to HDAC4 mRNA and interfere with its translation (Tuddenham *et al.*, 2006). Unfortunately, the biological relevance of these findings during bone development was not investigated. Nonetheless, these important studies opened a new line of investigation in the regulation of class IIa HDACs which deserves to be explored in the context of other class IIa HDACs-regulated developmental programs.

The existence of multiple regulatory pathways converging on class IIa HDACs emphasizes the importance of these enzymes in various biological processes. In the past 5 years, genetic inactivation studies in mice conducted by the Olson laboratory have been instrumental in elucidating the biological functions of these enzymes. These studies have revealed the key role of class IIa members in several important developmental and differentiation processes. Surprisingly, despite the large number of transcriptional regulators targeted by class IIa HDACs, most of their functions seem to involve the transcriptional repression of MEF2 transcription factors (Fig. 1). In the following sections, we will review the latest insights into the biological functions of these fascinating enzymes.

Chondrocyte hypertrophy

HDAC4-null mice die during the perinatal period due to severe growth retardations and numerous skeletal abnormalities that result from excessive hypertrophic chondrocyte differentiation and inadequate endochondral ossification (Vega *et al.*, 2004b). This remarkable phenotype was originally attributed to the ability of HDAC4 to repress Runt-related transcription factor-2 (Runx2), a well-recognized positive regulator of chondrocyte hypertrophy

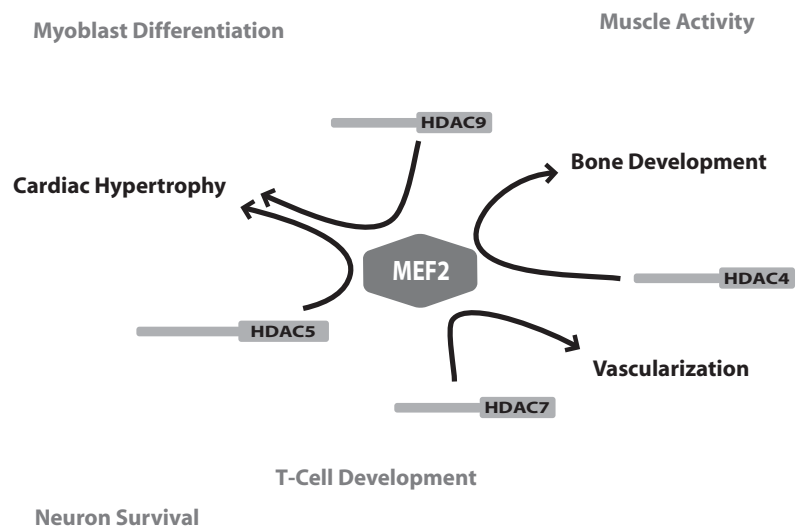


Fig. 1. Central role of MEF2 in the biological functions of class IIa HDACs. Gene targeting studies in mice have revealed tissue specific functions for each member of the class IIa HDACs. HDAC4-null mice display inappropriate chondrocyte hypertrophy whereas mice lacking HDAC7 die from cardiovascular defects and mutant mice for HDAC5 and HDAC9 develop spontaneous cardiac hypertrophy. Surprisingly, the known biological functions of class IIa HDACs have been associated with repression of the MEF2 family of transcription factors. A similar class IIa HDACs-MEF2 axis could be involved in cardiac muscle development, skeletal muscle differentiation and remodelling and T-cell apoptosis.

(Komori *et al.*, 1997; Otto *et al.*, 1997). Indeed, HDAC4 physically associates with Runx2 and inhibits its transcriptional activities. In addition, the phenotypic abnormalities of HDAC4 KO mice are strikingly reminiscent of those observed in mice with ectopic expression of Runx2 in prehypertrophic chondrocytes (Takeda *et al.*, 2001; Ueta *et al.*, 2001). The precise mechanism by which HDAC4 inhibits Runx2-mediated transcription remains obscure. Original observations suggested that repression of Runx2 by HDAC4 might occur independently of its HDAC catalytic activity. Instead, HDAC4 was thought to impede on Runx2 DNA binding by direct association with its Runt domain (Vega *et al.*, 2004b). However this model failed to explain why HDAC4 mutants consisting of the C-terminal catalytic domain of HDAC4, and thus lacking the Runx2 binding region, retain significant repressive activity. The recent observation that HDAC4 and HDAC5 deacetylate Runx2 and Runx3 provides an explanation for these apparent discrepancies (Jeon *et al.*, 2006; Jin *et al.*, 2004). In addition to preventing Runx2 from binding to DNA, HDAC4 may deacetylate Runx2 and promote its ubiquitin-mediated degradation. This double-targeting of Runx2 by HDAC4 would thus solely explain its key role as a negative regulator of chondrocyte hypertrophy.

This apparently satisfying model was recently challenged by a study published by the same authors and which unravelled the unexpected role of MEF2C in chondrocyte hypertrophy (Arnold *et al.*, 2007). This study reports that homo- or even heterozygous mutation of *Mef2c* is associated with severe skeleton defects resulting from reduced chondrocyte hypertrophy and ossification of endochondral bones. Given the preponderance of the class IIa HDACs-MEF2 axis in several developmental programs (see below), this unexpected finding raises the possibility that bone defects associated with deletion of *Hdac4* could, at least partly, result from hyperactivation of MEF2C. Supporting this hypothesis, a genetic antagonism exists between HDAC4 and MEF2: the excessive endochondral ossification observed in HDAC4 null mice is partially abolished by deletion of one *Mef2c* allele. Conversely, inactivation of *Hdac4* in the presence of a heterozygous *Mef2c* allele partially restored normal endochondral ossification. Interestingly, expression of Runx2 was greatly diminished in the endochondral cartilage of *Mef2c* mutant mice, indicating that some of the defects associated with *Mef2c* deficiency may be mediated by reduced Runx2 activity. By controlling key transcriptional regulators of chondrocyte hypertrophy, it is thus clear that HDAC4 plays a central role in the control of bone development. Interestingly, Runx2 is involved at multiple stages of bone development, such as osteoblast differentiation (Komori, 2008). Using *in vitro* models, HDAC7 was recently identified as a negative regulator of osteoblast differentiation through its Runx2 co-repressor function (Jensen *et al.*, 2008). In addition, HDAC4 and HDAC5 have been shown to participate in the repression of Runx2 by Smad3 during osteoblast differentiation (Kang *et al.*, 2005). While HDAC4 and -5 are expressed at significant levels in mesenchymal cells and osteoblasts, no evidence for HDAC7 expression in this cell lineage has been reported. In addition, the severe skeletal defects associated with HDAC4 deficiency were not observed in other class IIa HDACs mutant mice and could imply a specific role for HDAC4 as a negative regulator of bone development. A more careful examination of bone devel-

opment in mice lacking class IIa HDACs should unravel any functional overlap that may exist between these proteins.

Myogenesis

Skeletal muscle differentiation

Formation of skeletal muscle involves commitment of multipotential mesodermal precursor cells to the muscle lineage and their proliferation as myoblasts. Upon mitogens withdrawal, proliferating myoblasts exit the cell cycle and differentiate into multinucleated muscle fibers. The myogenic process results from a specific genetic program, with activation of hundreds of muscle-specific genes and repression of genes associated with cell proliferation. MEF2 has long been known as a key transcriptional regulator of skeletal muscle differentiation. Logically, the functional association between MEF2 family members and class IIa HDACs was originally tested in the context of myogenesis (Wang *et al.*, 1999; Miska *et al.*, 1999; Lu *et al.*, 2000a; Lemercier *et al.*, 2000). *In vitro*, class IIa HDACs negatively regulate muscle differentiation through association with MEF2 and repression of its target genes (Dressel *et al.*, 2001; Haberland *et al.*, 2007; Lu *et al.*, 2000b). In addition, the inhibitory action of class IIa HDACs can be overcome by myogenic signals that disrupt MEF2-HDAC interactions and stimulate nuclear export of these transcriptional repressors during muscle differentiation (McKinsey *et al.*, 2002). Surprisingly, normal overall skeletal muscle differentiation was maintained in mice mutated in each individual class IIa HDAC. This apparent discrepancy might result from the partial functional overlap that may exist between class IIa HDAC members. However, several observations indicate that the functional redundancy observed between class IIa HDACs *in vitro* may not exist *in vivo*. Indeed, class IIa members show distinct subcellular localizations in muscle cells. HDAC4 is mainly cytoplasmic in undifferentiated myoblasts and accumulates in the nucleus upon differentiation into myotubes, (Miska *et al.*, 2001). In contrast, HDAC5 and HDAC7 relocate from the nucleus to the cytoplasm as myoblasts differentiate into myotubes (Dressel *et al.*, 2001; McKinsey *et al.*, 2000a). These findings suggest that class IIa HDACs respond differently to physiologic stimuli and could thus have distinct roles during skeletal muscle differentiation.

Skeletal muscle remodelling

Skeletal muscle of adult vertebrates consists of type I and type II myofibers, which differ with respect to size, metabolism and contractile function. Slow-twitch or type I myofibers exhibit an oxidative metabolism and are resistant to fatigue, while in contrast, fast-twitch or type II fibers use glycolytic metabolism, fatigue rapidly and are involved in rapid bursts of activity. Numerous stimuli modulate skeletal muscle phenotype and induce switch from one specialized myofiber type to the other. MEF2 is preferentially activated in slow, oxidative myofibers (Wu *et al.*, 2000) and activates the slow myofiber gene program (Potthoff *et al.*, 2007). Logically, class IIa HDACs were recently implicated in the regulation of myofiber identity. In slow or oxidative fibers, class IIa HDACs are selectively degraded through a proteasome-dependent pathway. This specific degradation of class IIa HDACs alleviates their repression over MEF2, and probably other unidentified transcription factors allowing the induction of a slow skeletal muscle specific gene program. Mice lacking individual class IIa

HDACs in their skeletal muscle did not display abnormalities in fiber-type switching. However deletion of any combination of 4 alleles of *Hdac4*, -5, or -9 results in enhanced slow-fiber gene expression and increased percentage of slow myofibers, revealing functional redundancy between class IIa members. Interestingly, overexpression of constitutively active forms of PKD and CaMKIV, in adult glycolytic fibers of transgenic mice results in an increase in the number of slow fibers and enhances muscle oxidative capacity (Kim *et al.*, 2008a; Wu *et al.*, 2002). Because previous studies have established that these kinases stimulate MEF2 activity by promoting the phosphorylation and export of class II HDACs from the nucleus (Martin *et al.*, 2007), these observations further support a role for class IIa HDACs in fiber type specification.

In response to variations in environmental and functional demands, skeletal muscle adapt by remodelling the biochemical, morphological, and physiological states of individual myofibers (Bassel-Duby and Olson, 2006). These changes involve activation of intracellular signalling pathways and consequent genetic reprogramming, resulting in alterations of muscle mass, contractile properties, and metabolic states. Numerous stimuli, such as exercise, electrical stimulation or microgravity can induce muscle remodelling. Neuron-induced electrical activity has been known for a long time to induce specific transcriptional reprogramming of muscle and several neural activity-responsive genes have been identified. A typical example is the nicotinic acetylcholine receptors (AChRs), whose expression is highly sensitive to muscle innervation. AChR expression relies on the basic helix-loop-helix (bHLH) myogenic transcription factor myogenin. After innervation, neuron-induced electrical activity induces active transcriptional repression of myogenin throughout the muscle, which coincides with decreased extra-synaptic AChR expression levels. Recently, class IIa HDACs have been directly implicated in the regulation of myogenin expression. In innervated muscle, MITR, the non catalytic isoform of HDAC9, is highly expressed and represses transcription of myogenin through inhibition of MEF2. Conversely, denervation induces dramatic transcriptional downregulation of MITR, which correlates with subsequent myogenin and AChR expression (Mejat *et al.*, 2005). Interestingly, the observation that a MITR mutant protein lacking the MEF2-interacting domain retains 50% inhibitory activity on the expression of AChR and myogenin suggests that MITR could have additional targets besides MEF2. HDAC9-null mice do not show any obvious perturbation of skeletal muscle function under normal conditions but were sensitized to denervation. This sensitized phenotype is reminiscent of the cardiac phenotype observed in HDAC5 and HDAC9- null mice and establishes class IIa HDACs as general stress sensors and integrators.

Besides MEF2, Dash2, a Dachshund related transcriptional co-repressor whose expression is decreased upon denervation, was reported to inhibit myogenin expression in innervated muscle (Tang and Goldman, 2006). In contrast to MITR/HDAC9, HDAC4 expression is highly induced in response to denervation, suggesting that both class IIa HDACs could have opposite effects in denervation-induced nAChR expression (Cohen *et al.*, 2007). This model is supported by the observation that neuronal input also induces nuclear accumulation of HDAC4 (Liu *et al.*, 2005). In the nucleus, HDAC4 associates with the Dach2 promoter and thus activates the myogenin/AChR cascade through active down-

regulation of Dach2. Unexpectedly, the opposite regulations of HDAC4 and MITR by neuronal activity lead to the same transcriptional outcome: induction of myogenin/AChR expression by denervation. The most interesting corollary to these observations is that these two class IIa members must impede on the neuronal activity-dependent transcriptional reprogramming of muscle through non-redundant, complementary pathways. Whether similar combinatorial regulation by multiple class IIa members also exists for other transcriptional programs involving class IIa HDACs needs to be investigated.

Cardiac muscle development

Heart formation is initiated from the mesoderm in vertebrate embryos. The first stage of cardiomyogenesis involves the formation of cardiomyoblasts, which express a distinct subset of transcription factors, including MEF2C, the homeobox transcription factor Nkx2-5 and members of the cardiac GATA subfamily (Zaffran and Frasch, 2002). These factors activate the expression of cardiac-muscle-specific genes to form the differentiated cardiomyocytes. Overexpression of HDAC4, or inhibition of the inactivating class IIa HDAC kinase CaMK, was shown to inhibit the transition from mesoderm to cardiomyoblast during cardiomyogenesis in P19 cells (Karamboulas *et al.*, 2006b). Surprisingly, mice mutated for individual class IIa HDACs do not exhibit obvious abnormalities of the heart. In contrast, HDAC5/9 double mutant mice are prone to perinatal death from ventricular septum defects and thin ventricular walls, which typically originate from abnormalities in growth and maturation of cardiomyocytes (Chang *et al.*, 2004). Whereas the molecular mechanisms underlying the role of class IIa HDACs in these processes remain obscure, studies in mice and *Drosophila* have established the importance of MEF2 in the control of cardiac muscle differentiation (Bour *et al.*, 1995; Karamboulas *et al.*, 2006a; Lilly *et al.*, 1995; Lin *et al.*, 1997; Ranganayakulu *et al.*, 1995). It is thus likely that at least part of the cardiomyogenesis defects associated with class IIa HDACs inactivation arise from superactivation of MEF2 transcriptional activity. However, it is also possible that class IIa HDACs directly or indirectly repress other transcription factors that are important for cardiomyogenesis, such as SRF, CAMTA, Nkx2-5, myocardin and GATA factors (Davis *et al.*, 2003; Han *et al.*, 2006; Long *et al.*, 2007; Song *et al.*, 2006).

Cardiac hypertrophy

Cardiac hypertrophy, which is defined as an increase in cardiomyocyte size and reactivation of a specific fetal cardiac gene program, is an adaptive response of the heart to a variety of stress stimuli. Stress-induced hypertrophy may initially normalize ventricular wall stress, but excessive hypertrophic growth of the heart frequently leads to maladaptive changes that ultimately weakens cardiac performance and can lead to heart failure (Bucks and Olson, 2006; Hill and Olson, 2008).

Transcripts for every class IIa HDACs, including the splicing HDAC9 variant MITR are found at high levels in mouse heart (Zhang *et al.*, 2002). In addition, ectopic expression of constitutively repressive mutants of HDAC4, -5 and -9 (i.e. mutants insensitive to signal-responsive class IIa HDAC kinases) suppress hypertrophy of primary cardiomyocytes and expression of fetal cardiac genes *in vitro* (Bucks *et al.*, 2006; Vega *et al.*, 2004a; Zhang *et al.*, 2002). Gene-inactivation studies in mice have

provided definite evidence of the importance of class IIa HDACs as signal-responsive suppressors of postnatal cardiac growth. HDAC5 or HDAC9-KO mice exhibit no evidence of cardiac abnormalities during the early post-natal period. However, within their first year, these mutant mice develop spontaneous cardiac hypertrophy, which appears to result from hypersensitivity to age-related cardiac stress. HDAC5 and HDAC9 mutant mice also show exacerbated hypertrophic response to pressure overload or constitutive activation of calcineurin (Chang *et al.*, 2004; Zhang *et al.*, 2002). The remarkably similar cardiac phenotypes observed in HDAC5 or HDAC9 mutant mice suggest that these two class IIa members play redundant roles in the control of the same pathological hypertrophy signalling pathways. Unfortunately, mice lacking either HDAC4 or HDAC7 are not viable, which has precluded from investigating the potential involvement of these class IIa members in cardiac hypertrophy (Chang *et al.*, 2006; Vega *et al.*, 2004b). Conditional gene deletion studies will be needed to test whether HDAC4 and HDAC7 play functional roles similar to that of HDAC5 and HDAC9 in the cardiac pathological response.

MEF2, and in particular MEF2D plays an important role as an integrator and mediator of stress-dependent remodelling of the adult heart (Kim *et al.*, 2008b; Lu *et al.*, 2000a; Nadruz *et al.*, 2003). Indeed, *Mef2d*-null mice display impaired cardiac hypertrophic response to pressure overload and adrenergic signalling (Kim *et al.*, 2008b). Hypertrophic cardiac growth in HDAC5 or HDAC9 mutant mice correlates with superactivation of MEF2, suggesting that MEF2 is the critical target of class IIa HDACs in specific signalling pathways leading to cardiac hypertrophy. Indeed, while inactivation of *Mef2d* alleles renders mice resistant to cardiac remodelling in response to β -adrenergic stimulation, HDAC5 or HDAC9-null mice respond normally to isoproterenol stimulation (Chang *et al.*, 2004; Kim *et al.*, 2008b). This interesting observation could indicate that only specific signalling pathways of cardiac hypertrophy involve class IIa HDAC-mediated regulation of MEF2. On the other hand, the insensitivity of mice lacking either HDAC5 or HDAC9 to adrenergic stimuli could suggest a functional redundancy with other class IIa members in this particular pathway. Identification of the precise role and specific physiological relevant targets of each class IIa HDAC in cardiac hypertrophy will help solving these important issues.

Neuronal survival

HDAC4 and HDAC5 mRNA are highly abundant in the brain suggesting a role for these class IIa members in neurons (Grozinger *et al.*, 1999). HDAC4 is predominantly cytoplasmic in cerebellar granule neurons (CGNs), and signals promoting its nuclear translocation or expression of constitutively nuclear mutants correlates with apoptosis (Bolger and Yao, 2005). Similarly, HDAC5 is mainly cytoplasmic in CGNs cultured under depolarizing conditions. Switching CGNs to non-depolarizing medium, which induces cytoplasm-to-nucleus translocation of HDAC4 and -5, correlates with induction of apoptosis (Linseman *et al.*, 2003). MEF2 is a well-established survival factor in neurons (Mao *et al.*, 1999) and as expected, induction of apoptosis by HDAC4 and -5 correlates with repression of MEF2 transcriptional activity (Bolger and Yao, 2005; Linseman *et al.*, 2003). However, whether HDAC4 and/or HDAC5 target any other transcription factor besides MEF2 remains to be explored. Interestingly, HDAC4 appears to have a

more predominant role in neuronal cell death, as siRNA-mediated inhibition of HDAC4 efficiently suppresses neuronal death, arguing against functional redundancy between HDAC4 and HDAC5 (Bolger and Yao, 2005). In addition, HDAC5 knock-down mice showed no obvious brain defects, whereas mice lacking HDAC4 have abnormal shaped brains and exencephaly, probably resulting from precocious ossification of the brain (Vega *et al.*, 2004b). However, more careful examination of HDAC4-null mice revealed delay in the formation of folia, suggesting a pro-survival rather than a pro-apoptotic role for HDAC4 (Majdzadeh *et al.*, 2008; Vega *et al.*, 2004b). Indeed, a recent study reported that HDAC4 prevents low-potassium induced neuronal cell death, through inhibition of activity of cyclin-dependent kinase-1 (CDK1). Supporting their results, the authors described higher CDK1 activity in the brain of HDAC4^{-/-} mice (Majdzadeh *et al.*, 2008). Similarly, studies using a model of mouse retina degeneration showed that HDAC4 promotes survival of photoreceptor cells (Chen and Chepko, 2009). Interestingly, this pro-survival function of HDAC4 was associated with its presence in the cytoplasm and might thus be independent of its nuclear repressive function. The reasons for these contradictory results remain unclear and should instigate further studies about the role of class IIa HDACs in neurons.

Immune cells

After entering the thymus, precursor T-cells differentiate into mature lymphocytes following a complex and highly regulated process (Ciofani and Zuniga-Pflucker, 2007). Complete maturation of developing thymocytes implies that they successfully pass a series of developmental checkpoints. After complete rearrangement of their T-cell receptor (TCR) chains, thymocytes become CD4⁺/CD8⁺, or double-positive (DP) thymocytes and undergo negative and positive selection. During these processes, their TCR is tested for its ability to interact with self-peptide bound to major histocompatibility complex (MHC) molecules of the thymic antigen-presenting cells (APCs). Positive selection will only allow further differentiation of thymocytes carrying a functional TCR. Potentially autoreactive thymocytes bearing TCR with strong affinity to MHC-self peptide complexes are deleted by the apoptotic process of negative selection. The different developmental fates of maturing thymocytes are dictated by the integration of various signals and the translation of these signals into changes in gene expression.

In human, HDAC7 is highly expressed in thymus, heart and lung. Within the thymus, we have shown that HDAC7 is transiently and predominantly expressed in CD4/CD8 DP thymocytes (Dequiedt *et al.*, 2003). Consistent with this, HDAC7 associates with MEF2D and represses the expression of a series of MEF2-target genes involved in negative and positive selections, such as the pro-apoptotic orphan nuclear receptor *Nur77* (Dequiedt *et al.*, 2003; Kasler and Verdin, 2007). The release of HDAC7-mediated repression is achieved through a complex signalling cascade. Engagement of the TCR by MHC-self peptides results in dissociation of HDAC7-MEF2D complexes, phosphorylation and cytoplasmic accumulation of HDAC7 which ultimately leads to de-repression of MEF2-target genes (Dequiedt *et al.*, 2005). Interestingly, microarray-based

analysis has revealed that HDAC7 could regulate both MEF2-dependent and independent genes (Kasler and Verdin, 2007). However, how HDAC7 regulates transcription independently of MEF2 was not investigated. Amongst the MEF2-HDAC7 regulated genes in thymocytes, this study also identified HDAC5, which could suggest a role for this class IIa member during T-cell differentiation.

In B cells, signalling from the B-cell antigen receptor has been shown to regulate HDAC5 and HDAC7 phosphorylation, localization and repressive function (Matthews *et al.*, 2006). Even if chemical inhibition of HDACs supports a role for HDACs in B-cell differentiation and survival, the transcriptional targets and functional significance of these findings remain currently unknown.

Vascularisation

Early studies using general HDAC inhibitors suggested the possible implication of zinc-dependent HDACs in the transcriptional control of endothelial gene expression and vascular development (Kim *et al.*, 2001; Kwon *et al.*, 2002). However, until recently, definite experimental evidence about the function of class I and class II HDACs in these processes was lacking.

In situ hybridization studies revealed specific expression of HDAC7 in the vascular endothelium during mouse embryogenesis. Consistent with this, inactivation of the HDAC7 gene in a ubiquitous or endothelial specific manner had dramatic consequences on vascular integrity, with mutant mice dying *in utero* from blood vessel dilatations, ruptures and haemorrhages. In addition, siRNA-mediated inactivation of HDAC7 in human umbilical vein endothelial cells (HUVECs) prevents formation of capillary-like structures *in vitro* (Chang *et al.*, 2006). Microarray analysis revealed that HDAC7 regulates the expression of several genes encoding extracellular matrix and adhesion proteins, among which the secreted matrix metalloprotease 10 (MMP-10). Regulation of *MMP-10* expression by HDAC7 relies on its association with MEF2C, a factor implicated in blood vessel development and vascular integrity (Lin *et al.*, 1998), and constitutes yet another example in which the class IIa HDACs-MEF2 axis controls an important genetic developmental program. Inappropriate expression of MMP10 in HDAC7-null mice would be expected to disrupt endothelial cell-cell adhesion and could thus explain the defects associated with HDAC7 inactivation. However, a more recent study has brought some new light on the role of HDAC7 during angiogenesis and vascularisation. Using a complete set of *in vitro* assays, Mottet and colleagues have shown that the inability of HDAC7-silenced HUVECs to assemble into tube-like structures *in vitro* could result from alterations of their motility (Mottet *et al.*, 2007). In addition, HDAC7 silencing correlates with increased expression of PDGF-B, a regulator of migration and tubulogenesis of endothelial cells (De Marchis *et al.*, 2002). Even though the molecular mechanisms underlying the transcriptional control of PDGF-B by HDAC7 are unknown, these findings raise the appealing possibility that HDAC7 might regulate multiple stages during vasculature formation. Interestingly, HDAC7 silencing was also associated with altered morphology of the cells, which could also impact on their angiogenic properties (Holderfield and Hughes, 2008). Considering its potential impact on multiple

aspects of vasculogenesis, identification of HDAC7 target genes has become a necessary step toward a better understanding of vascular development.

Future prospects

As described above, the past 5 years have witnessed the emergence of class IIa HDACs as key regulators in several important biological processes. Through their signal-responsive regulation and their ability to repress the transcriptional activity of numerous transcription factors, class IIa HDACs integrate and translate extracellular signals into specific genetic activation programs. Recent studies in mice deficient for each class IIa member have corroborated this model and revealed major defects in important developmental programs such as embryonic bone formation and vascularisation and cardiac hypertrophy (see above). While groundbreaking, these studies have focused on the functions of class IIa HDACs leading to the most spectacular phenotypical defects. However, the early observation that class IIa HDACs are expressed in multiple tissues suggests that they might have several biological functions, depending on the cellular context. More careful examination of class IIa HDAC null-mice should identify less obvious phenotypical defects and therefore suggest additional biological functions for these enzymes. For instance, while HDAC4-deficient mice die perinatally from skeletal defects, demonstrating the key role of this class IIa member in bone development, they also exhibit cerebellar abnormalities which could support a role for HDAC4 in neuronal survival (Majdzadeh *et al.*, 2008).

Surprisingly, and despite the fact that they can associate with a plethora of partners, the biological functions of class IIa HDACs seem to rely almost uniquely on their association with MEF2 transcription factors. MEF2 has been implicated in a myriad of developmental, physiological and pathological processes, each of which could potentially be regulated by a class IIa HDACs-MEF2 axis. Amongst the MEF2-regulated processes, the unique or redundant *in vivo* functions of class IIa members remain to be investigated in contractile myofibril assembly (Hinits and Hughes, 2007), craniofacial development (Verzi *et al.*, 2007), postsynaptic morphogenesis and brain development (Shalizi *et al.*, 2006; Shalizi and Bonni, 2005). Inversely, several lines of experimental evidence suggest that class IIa HDACs can regulate transcription independently of MEF2 (Kasler and Verdin, 2007). Nonetheless, whether MEF2-independent biological functions exist for class IIa HDACs remains an unanswered question and deserves to be thoroughly addressed. The existing studies about the *in vivo* functions of class IIa HDACs have overlooked the many other transcriptional targets of these enzymes and often linked the observed physiological defects with hyperactivation of MEF2. The existence of knock-out mice for each class IIa HDAC member gives now the opportunity to validate additional known class IIa HDAC partners besides MEF2.

Functional inactivation of class IIa HDACs via signal-dependent phosphorylation and nuclear export has been extensively illustrated in *in vitro* systems (Martin *et al.*, 2007). This mechanism is now being validated *in vivo* by recent studies showing the importance of known class IIa HDAC kinases in cardiac hypertrophy (Backs *et al.*, 2006; Fielitz *et al.*, 2008; Harrison *et al.*, 2006) and skeletal muscle function and remodeling (Kim *et al.*, 2008a).

Logically, class IIa HDAC kinases should also be involved in other class IIa HDAC regulated genetic programs such as chondrocytes hypertrophy, neuron and immune cells apoptosis (Dequiedt et al., 2005; Parra et al., 2005), and blood vessel development (Harrison et al., 2006).

The establishment of class IIa HDACs as key modulators of various differentiation and developmental programs, especially in pathological related processes, potentially offers new therapeutic strategies for various human diseases. A still limited number of studies have reported alterations of class IIa HDACs in several cancers (Chaabouni et al., 2006; Ouaisi et al., 2008; Ozdag et al., 2006; Yuki et al., 2004) and neuronal disorders (Hoshino et al., 2003; Iga et al., 2007; Renthal et al., 2007). Armed with the new insights into their regulation and biological functions, it seems possible to specifically modulate class IIa HDAC activity and normalize pathological gene expression patterns in disorders such as cardiac hypertrophy, heart failure, tumour growth or skeletal muscle wasting.

Acknowledgements

We apologize to those investigators whose work was not cited in this article owing to space limitations. This work was supported by the Belgian National Fund for Scientific Research (F.N.R.S) and the Interuniversity Attraction Poles Programme, Belgian Science Policy (PAI6/07). F.D. is Research Associate, M.M. is Research Fellow and R.K. is Research Director of the F.N.R.S.

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