Post-translational Modifications Regulate Class IIa Histone Deacetylase (HDAC) Function in Health and Disease*[S]

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Class IIa histone deacetylases (HDACs4, -5, -7, and -9) modulate the physiology of the human cardiovascular, musculoskeletal, nervous, and immune systems. The regulatory capacity of this family of enzymes stems from their ability to shuttle between nuclear and cytoplasmic compartments in response to signal-driven post-translational modification. Here, we review the current knowledge of modifications that control spatial and temporal histone deacetylase functions by regulating subcellular localization, transcriptional functions, and cell cycle-dependent activity, ultimately impacting on human disease. We discuss the contribution of these modifications to cardiac and vascular hypertrophy, myoblast differentiation, neuronal cell survival, and neurodegenerative disorders. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.046565, 456–470, 2015.***

The enzymatic addition of acetyl moieties to lysine residues and the removal of these modifications via deacetylation were first documented over forty years ago (1, 2). As histones were the first substrates shown to be modified by acetylation (2), the enzymes responsible for adding and removing acetylations are commonly known as histone acetyltransferases and histone deacetylases (HDACs)[1], respectively. HDACs have been shown to regulate a multitude of cellular processes, including cell cycle progression, differentiation, and apoptosis (3–5). In particular, HDACs have been intensively studied for their abilities to control gene transcription and to manipulate the epigenetic status of cells (6). HDACs are generally thought to promote transcriptional repression and gene silencing (7–9); however, nonhistone proteins are also targeted by these enzymes, and include cell receptors, chaperones, and cytoskeletal proteins (10). To date, 18 human HDACs have been identified and categorized into four classes based on homology to yeast deacetylases (11). Class I (HDAC1, -2, -3, and -8) are related to Rpd3, class IIa (HDAC4, -5, -7, and -9) and class IIb (HDAC6 and 10) are similar to Hda1, and class IV (HDAC11) has homology to both Rpd3 and Hda1 (11). These HDAC classes are zinc-dependent enzymes, in contrast to class III HDACs, commonly known as sirtuins (SIRT1–7), which require the cofactor NAD+ for activity (12, 13).

In recent years, the class IIa HDACs have been established as critical regulators of numerous cellular processes (14, 15), with misregulation being associated with a wide range of human diseases. Roles for class IIa HDACs have been reported in the development of cardiovascular disease (16), cancer (17), immune response to viral infection (18), epigenetic response to drug stimulus (19), diabetes (20), and neurodegenerative diseases, such as Huntington's disease (21). Intriguingly, class IIa HDACs have been shown to limit intrinsic enzymatic activity. Their repressive activity is proposed to depend largely on their association with class I enzymes, in particular HDAC3, and on their ability to repress transcription factors in a deacetylation-independent manner. Structurally, the limited activity of class IIa HDACs is the result of an amino acid substitution (His) at the catalytic Tyr present in the DAC domains of other eukaryotic and prokaryotic deacetylases. Indeed, His-to-Tyr mutation of HDAC4 resulted in elevated (1000×) enzymatic activity compared with wild type (22). Additionally, the identification of a zinc binding region, containing a CCHC motif, within class IIa HDACs suggests the possibility that this and other regions could be important for substrate recognition, binding, and regulation of HDAC catalytic activity because of proximity to the active site (23). However, in contrast to the better understanding of class I HDAC regulation and functions (24), the mechanisms governing class IIa HDAC functions in health and disease states still remain less well understood.

It has recently become increasingly clear that class IIa HDAC functions are significantly regulated by post-translational modifications (PTMs), including phosphorylations, acetylations, sumoylations, and ubiquitinations (25). PTMs

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[1] The abbreviations used are: HDAC, histone deacetylase; PTM, post-translational modification; NES, nuclear export sequence; NLS, nuclear localization sequence; MEF2, myocyte enhancement factor; CaMK, calcium/calmodulin dependent kinase; PP2A, protein phosphatase2A; PKA, protein kinase A; PKD, protein kinase D; MITR, MEF2 interacting transcription repressor; NCoR, nuclear co-repressor.

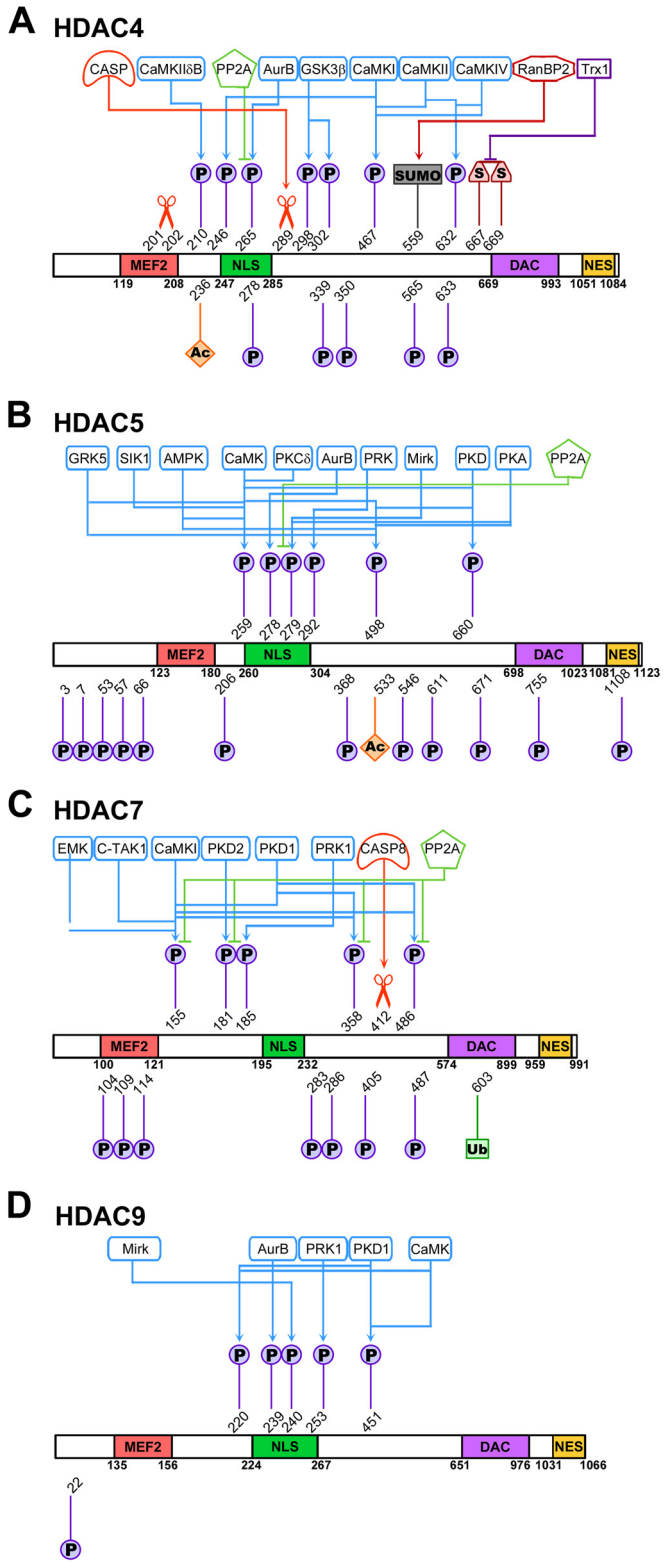

FIG. 1. **Numerous signaling pathways converge to post-translationally modify class IIa HDACs.** Class IIa HDACs are modified by phosphorylation, sumoylation, disulfide bridges, acetylation, ubiquitinylation, and are cleaved by caspases. Sites with enzymes assigned to their modification are shown at the top of each protein schematic; uncharacterized sites are shown at the bottom. Functional domains of class IIa HDACs are highlighted: MEF, MEF2 binding domain; NLS, nuclear localization signal; DAC, deacetylation domain; NES, nuclear export sequence. *A*, HDAC4, *B*, HDAC5, *C*, HDAC7, *D*, HDAC9.

provide important dynamic points of regulation for determining protein structure, protein interactions, and protein localizations, and additionally, in the case of HDACs, their transcriptional repressive functions (26–28). Given the recent expansion in the knowledge of numerous PTMs on class IIa HDACs, an ongoing challenge is to collate this meaningful information and identify key residues that elicit precise physiological functions. This review will first construct a comprehensive map of site-specific class IIa HDAC PTMs and the enzymes that regulate them. The functional significance of individual PTMs will then be discussed relative to their intracellular roles and their contributions to disease progression.

Site-specific Post-translational Modifications of Class IIa HDACs—Diversification of the human proteome can be achieved at the post-transcriptional level through mRNA splicing (29) and post-translational modification of polypeptides (30). A protein can be "decorated" with such chemical modifications that alter its activity and functions. For class IIa HDACs, the functions of these specific PTMs are critically associated with their location within the protein domains. HDAC4, -5, -7, and -9 possess a definitive bipartite structure (31). The C terminus contains a conserved deacetylase domain and a hydrophobic nuclear export sequence (NES), which promotes cytoplasmic accumulation (32) (Fig. 1). The N-terminal region, or adaptor domain, contains a lysine/arginine-rich nuclear localization sequence (NLS) and binding sites for transcription factors, such as the myocyte enhancer factor-2 (MEF2) (33, 34), and chaperone proteins (35–37). These domains serve as platforms for the formation of higher order protein complexes, such as by interactions with the C-terminal-binding protein and heterochromatin P1, through which HDACs perform specific enzymatic and functional activities (31). HDAC association with chaperone proteins enables their nuclear export and relieves repression of HDAC target genes (35–37) (discussed in "PTMs Regulate HDAC Subcellular Localization and Orchestrate Transcription"). This section will review the current knowledge of site-specific modifications, as integrated from comprehensive literature searches and from multiple databases, highlighting their functional relevance and regulation by distinct enzymes (Fig. 1).

HDAC4—HDAC4 is the most well-characterized member of the class IIa family in terms of the number of identified PTMs, which include phosphorylation, acetylation, sumoylation, and disulfide bond formation (Fig. 1*A*). Furthermore, many of the enzymes regulating these modifications have been characterized. The numerous phosphorylations on HDAC4 provide a platform for finely tuned control of protein functions, as each modification can exert a function as a single signal, or can act in tandem with other modifications (supplemental Table S1).

Phosphorylations at S246, S467, and S632 initiate binding of 14-3-3 chaperone proteins that promote shuttling of HDAC4 to the cytoplasm (35, 37). As HDAC localization is tightly connected to transcriptional regulatory function, loss of phosphorylation enhances MEF2 expression by spatially excluding HDAC4 from the nucleus rather than by limiting its intrinsic enzymatic activity (35, 37).

Several isoforms of the calcium/calmodulin-dependent kinase (CaMK) family phosphorylate HDAC4 (38–46) and regulate its ability to repress target genes in the nucleus (supplemental Table S1), suggesting a high degree of regulatory specificity with respect to individual sites. CaMKI preferentially phosphorylates S246 on HDAC4, having only limited activity toward S467 and S632 (40). The major regulatory kinase of S467 is CaMKII, which is also able to efficiently phosphorylate S632 (40) (Fig. 1*A*). S467 and S632 are also known to be phosphorylated by CaMKIV and can promote nuclear export by a mechanism independent of 14–3-3 binding (39). CaMKIIδ_B is also known to preferentially target S210 in cardiac cells (41), and this modification is associated with heart disease (see "The Post-translational Regulation of HDACs is Critically Linked to Human Disease").

Apart from the CaMK superfamily, a diverse set of enzymes is involved in regulating HDAC4 modifications. Although the regulation of some sites remains unknown, the S265 and S266 NLS sites were shown to be phosphorylated by Aurora B and Mirk/dyrk1B, respectively (47, 48). S298 and S302 phosphorylation status are both under the control of the glycogen synthase kinase 3β (GSK3β), and the phosphomimetic S298D mutation promotes polyubiquitination of HDAC4, suggesting a link between phosphorylation and ubiquitin PTMs (49). The proteins and pathways involved in the dephosphorylation of HDAC4 have also been investigated. Several studies have shown that the protein phosphatase 2A (PP2A) family can remove HDAC4 phosphorylation, promoting its nuclear accumulation (50–53).

Additional PTMs modulate the subcellular localization of HDAC4 independently of its phosphorylation status. Hypertrophic stimuli promote the oxidation of C667 and C669 and the formation of a disulfide bond that signals HDAC4 nuclear export (54). Conversely, sumoylation of HDAC4 by the E3 ligase RanBP2, which covalently attaches SUMO-1 to HDAC4 at K559, promotes nuclear retention (55). Caspase-mediated cleavage can also enhance HDAC4 nuclear accumulation. caspase 2- and caspase 3-dependent cleavage of HDAC4 at D289 generates an NLS-containing protein fragment that represses transcription factors and activates apoptotic pathways (56). HDAC4 cleavage is also linked to protein kinase A (PKA)-mediated processing that leads to the repression of MEF2 activity (57). Cleavage occurs between T201 and W202 to release a ~28 kDa N-terminal fragment; however, as no PKA phosphorylation sites were determined for HDAC4, the association between PKA, HDAC4, and the protease requires further investigation (57).

HDAC5—Although numerous HDAC5 PTMs have been identified in recent years, fewer of these modifications have been functionally characterized. Those that have been investigated are clustered within and around the NLS (Fig. 1*B*, *top*). However, proteomic investigation of HDAC5 has also revealed extensive PTMs throughout the protein sequence, including within the DAC and NES domains (28) (Fig. 1*B*, *bottom*). Through a mechanism conserved across all class IIa HDACs, critical HDAC5 residues (S259 and S498) promote 14–3-3 chaperone protein binding (27, 36) and nuclear export upon phosphorylation by CaMK-I, -II, and -IV (27, 43, 58, 59). Distinct from analogous HDAC4 sites, these key residues are known to be phosphorylated by additional kinases (Fig. 1*B*, supplemental Table S1). Regulation of HDAC5 by protein kinase D (PKD) and its various isoforms has been widely reported (*e.g.* (60–65)). PKD also phosphorylates S660, an additional 14–3-3 binding site (66). PKC, an upstream regulator of PKD, can also phosphorylate S259 directly (67), whereas PKA can modify S498 (66). PKC-related kinases 1 and 2 (PRK1 and PRK2) also target T292 (68), highlighting the PKC signaling pathway as an important determinant of HDAC5 phosphorylation status. Other enzymes that target both S259 and S498 are AMP-activated protein kinase (69, 70), G protein-coupled receptor kinase-5 (GRK5) (71), whereas salt-inducible kinase 1 (SIK1) phosphorylates only S259 (72).

Recent investigation of phosphorylations within the NLS has uncovered new regulatory features of HDAC5. S279 phosphorylation has been shown to be critical for the nuclear HDAC5 localization (28) (discussed in "PTMs Regulate HDAC Subcellular Localization and Orchestrate Transcription") (supplemental Table S1). Mirk/dyrk1B (48), PKA (73), and Cdk5 (74) have all been shown to phosphorylate S279, whereas PP2A has been reported to dephosphorylate it (74). Interestingly, the phosphorylation of the immediately adjacent NLS serine residue, S278, was shown to have a distinct spatial and temporal regulation, being controlled by Aurora B in a cell cycle-dependent manner (47). NLS phosphorylation by Aurora B is conserved in both HDAC4 and HDAC9.

HDAC7—Readers should note that HDAC7 amino acid numbering has changed considerably over the years as sequence information has been updated. To limit ambiguities, the sites we discuss are consistent with those reported in the PhosphoSitePlus database (Fig. 1*C*); please note that in some studies sites 179, 344, and 479 correspond to 155, 358, and 486 (supplemental Table S1). Similar to other class IIa HDACs, phosphorylation regulates the interaction between HDAC7 and chaperone proteins and protein stability (75). The HDAC7 residues critical for these processes are S181, S155, S358, and S486 (76). The latter three sites can be modified by CaMKI (77, 78), whereas PKD can phosphorylate all four residues (76, 79–83). In addition, hPar-1 kinases EMK and C-TAK1 phosphorylate S155 (84), and PRK1 targets S185 (68). The modification of individual sites by multiple distinct

kinases demonstrates that phosphorylation is a finely tuned system for regulation of HDAC function. Furthermore, the ability of a diverse set of kinases to regulate the same sites may reflect an evolutionary flexibility that allows differential modulation of critical functional sites. Interestingly, dephosphorylation of the four 14–3-3 binding sites in HDAC7 is mediated by PP2A, which shows no preference for one site over the others (85). Although the precise sites of action remain unknown, protein phosphatase 1β and myosin phosphatase targeting subunit 1 promote nuclear accumulation of HDAC7 (86). Like HDAC4, the stability of HDAC7 can be mediated though caspase-dependent cleavage. Caspase-8 cleaves HDAC7 at D412 (87), generating an unstable N-terminal fragment that resides in the nucleus and a C-terminal fragment that is excluded from the nucleus (88).

HDAC9—HDAC9 remains the least well-characterized class IIa HDAC in terms of known PTMs and modifying enzymes (Fig. 1*D*). Information about HDAC9 has been derived based on its high degree of homology to the other class IIa family members. Prior to the cloning of HDAC9 (89), an isoform lacking the DAC domain and consisting of residues sharing homology with HDAC4 and HDAC5 was identified and named MEF2-interacting transcription repressor (MITR) (34). The sites regulating 14–3-3 binding in HDAC5 (27) were observed to be conserved in MITR at S220 and S451. Moreover, phosphorylation by CaMK disrupted MEF2 interaction and altered the localization of MITR (90). Later, PKD was also shown to phosphorylate these residues (81), modifications also detected in cortical neurons (91). Additionally, HDAC9 residues S239, S240, and S253 are phosphorylated by Aurora B (47), Mirk/dyrk1B (48), and PRK1 (68), respectively (Fig. 1*D*), similar to the analogous residues in other class IIa HDACs.

The Distribution of PTMs within HDAC Structure—The positioning of PTMs within different structural features of HDACs can significantly contribute to determining their functions. As detailed previously, the PTMs within or in the proximity of the NLS have been well documented to modulate HDAC localization and transcriptional repressive function. However, the presence of PTMs within multiple HDAC functional domains and regions with distinct structural properties must also be considered. To examine the distribution of HDAC PTMs (red stars, Fig. 2), disordered protein regions were predicted using the PrDOS software (92). Established domains, including the MEF binding, NLS, DAC, and NES, exhibit varying degrees of disorder, with the DAC domain representing the least disordered region of each HDAC. The highly structured prediction for the DAC domain is not surprising, given that the first crystal structure solved for class IIa HDACs was the catalytic domain of HDAC7 (23). Highly ordered regions may be favorable environments for structural PTMs, such as the disulfide bond that forms immediately adjacent to the DAC domain in HDAC4 (Figs. 1 and 2). The MEF domain itself harbors no currently known PTMs, but appears to have greater structural variation across the class IIa HDAC family than the NLS,

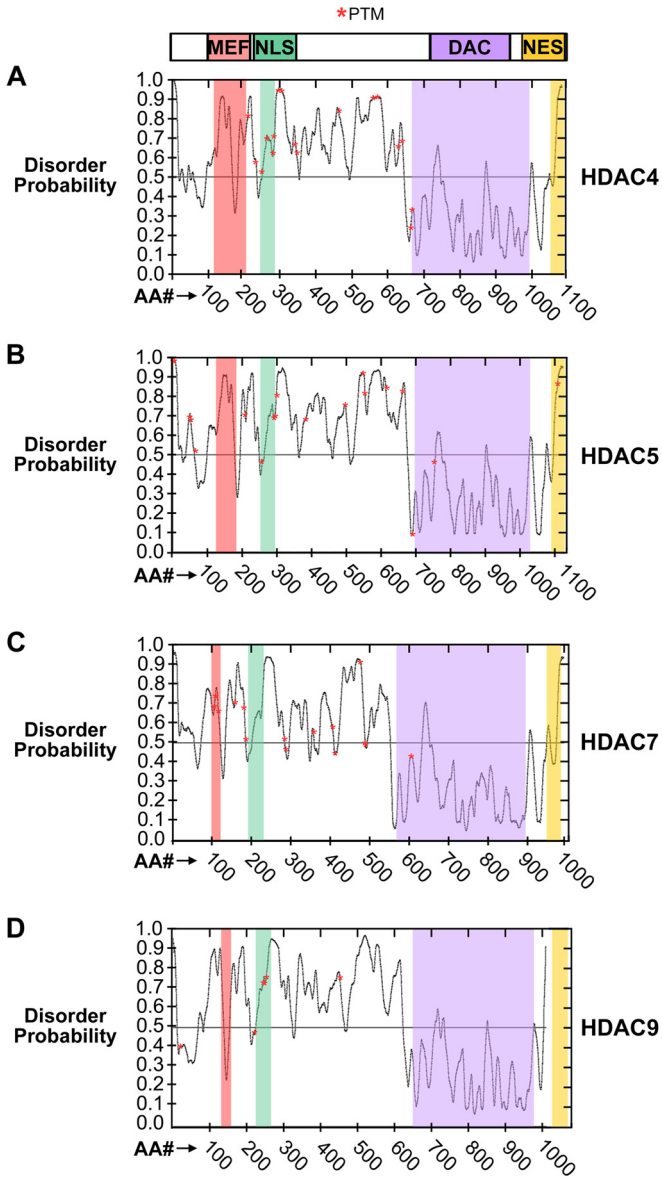

FIG. 2. **The distribution of class IIa HDAC post-translational modifications within ordered and disordered regions.** The probability of disorder was determined for each HDAC amino acid sequence using the PrDOS prediction software. Known PTMS are represented as red stars. Regions falling within established HDAC functional domains are shaded: MEF, red; NLS, green; DAC, purple; NES, yellow. *A*, HDAC4 sequence, *B*, HDAC5 sequence, *C*, HDAC7 sequence, *D*, HDAC9 sequence.

suggesting a possible source of plasticity in transcription factor binding preferences among HDACs. Interestingly, phosphorylations found in the NLS appear to map to the more disordered portion of this region (Fig. 2).

Previous HDAC5 structure prediction indicated that the majority of phosphorylations map to the external surface of the protein, suggesting that these modifications may be primarily important for mediating protein interactions (12). Loss of phosphorylation within an acidic region, the DAC domain, and

the NES was shown to modulate HDAC5 protein interactions, including associations necessary for transcriptional repression (12). Interestingly, mass spectrometry-based quantification of HDAC5 phosphorylations within these functional domains showed substantial variation in their relative abundances (12). Prominent modifications could point to sites necessary for protein folding or structural stability, whereas modifications with low abundances may reflect dynamic or transient events that are spatially and temporally modulated. It may be of interest to investigate possible correlations between the degree of order surrounding a PTM and its abundance.

Most HDAC PTMs seem to localize within regions predicted as naturally disordered (Fig. 2), in agreement with previous reports of abundant phosphorylation within disordered regions of other proteins (93). These regions are likely to represent more rapidly evolving sequences, and it is estimated that a significant percentage of S/T/Y sites (~25%) within disordered regions are modified (29). The increased availability of these sites may also promote their regulation by multiple enzymes, thereby allowing flexible control over protein function without altering amino acid sequences and conferring an evolutionary advantage. Indeed, numerous sites on class IIa HDACs are targeted by multiple enzymes (Fig. 1) and under different biological conditions (supplemental Table S1).

Although it is clear that numerous PTMs play important roles in regulating protein functions, it is also possible that a subset of PTMs occur from random subcellular events. Although enzymatic post-translational modification does require energy input, additions could be energetically favorable within local reaction environments of the densely packed cell. Thus, expending additional energy to prevent their occurrence or to actively remove PTMs would be of no great benefit when these PTMs do not impact protein function. Another likely explanation for yet-uncharacterized PTMs is that the appropriate functional assays have not yet been discovered or employed. Individual PTMs may exert their functions at specific time points or locations. Currently, as little is known about the co-occurrence of multiple PTMs on HDACs at any given time, it would be of significant interest to examine the coordinated regulation of PTMs and the possibility of synergistic modification. Multiple sites await detailed site-specific investigation of their biological functions, which will no doubt be of tremendous value in building a more complete understanding the regulation of HDACs functions. For these yet functionally uncharacterized sites, we direct interested readers to the following papers that reported their discovery: HDAC4-K236 (94), T278 (95), S339 (96), S565 (97–99), S633 (97); HDAC5-(S3, S7, S53, S55, S66, S206, T234, S368, S755) (28), K533 (100), T546 (101), S671 (28, 102), S1108 (28, 103); HDAC7-T104 (104), S109 (97, 104), Y114 (105), S283 (97, 106), T286 (97, 106), S405 (102), S487 (107, 108), K603 (109), and HDAC9-S22 (96). The remainder of this review will focus on PTMs that have been functionally characterized and shown to regulate subcellular localization and transcription, thereby significantly contributing to maintenance of health and disease states.

PTMs Regulate HDAC Subcellular Localization and Orchestrate Transcription—Class IIa HDAC PTMs profoundly impact their capacity to modulate transcription of gene targets. Within the nucleus, HDACs carry out their transcriptional repressive functions as components of numerous multiprotein complexes (10), including the nuclear corepressor (NCoR) (110–112) and the Bcl-6 corepressor complexes (113). The nuclear association of class IIa HDACs to transcription factors through their N termini and to corepressors through their C termini provides a mechanism of targeting these specific functional protein complexes to particular genomic regions. The deacetylation of targets themselves is performed through association with various corepressor complexes that contain a class I HDAC (*i.e.* predominantly HDAC3), such as C-terminal-binding protein (114), NCoR/SMRT (110), and BCoR (115). For HDAC4, HDAC5, and HDAC9, the interaction with the E1A C-terminal binding protein occurs through a conserved N terminus PxDLS-like motif (114). The NCoR/SMRT complexes have been shown to associate with and regulate multiple transcription factors, including PLZF (116), BCL6 (117), MyoD (118), and Bach2 (119). The interaction with the SMRT complex was mapped to the C-terminal deacetylation domain of HDAC5 and HDAC7 and domains III and IV of SMRT (111, 120). NCoR and SMRT are also known to interact with HDAC3, as well as the HDAC1/HDAC2-containing Sin3 repressor complex (121). HDAC7 also interacts directly with mSin3A via an amphipathic helix in its N terminus (111), pointing to the complex regulation of HDAC associations with corepressors through both direct and indirect mechanisms. Another important nuclear interaction of HDAC4, HDAC5, and HDAC9 is with heterochromatin protein 1, which represses transcription via recruitment of histone methyltransferases, thus, linking the functional roles of deacetylation and methylation in regulating gene expression (122).

As mentioned previously, phosphorylation is a well-established regulator of HDAC protein localization, impacting the association with these distinct functional complexes (Fig. 3*A*). Phosphorylation of sites flanking the NLS in all class IIa HDACs promotes 14–3-3 chaperone protein binding and subsequent nuclear export in a CRM1-mediated fashion (27, 33, 78, 90, 123). Specifically, CRM1 binds a hydrophobic C-terminal NES motif conserved among class IIa HDACs (123). Association of 14–3-3 proteins was initially discovered for HDAC4 and HDAC5 using yeast two-hybrid and protein co-immunoprecipitation studies (35, 36). In the same study, HDAC4 and HDAC5 were also shown to associate with the Class I enzyme HDAC3 (35); recent quantitative proteomic studies have highlighted that this association occurs via binding to NCoR components (10). Although 14–3-3 simultaneously recognizes and binds to multiple phosphorylated sites, phosphorylation of a single site is sufficient for maintaining

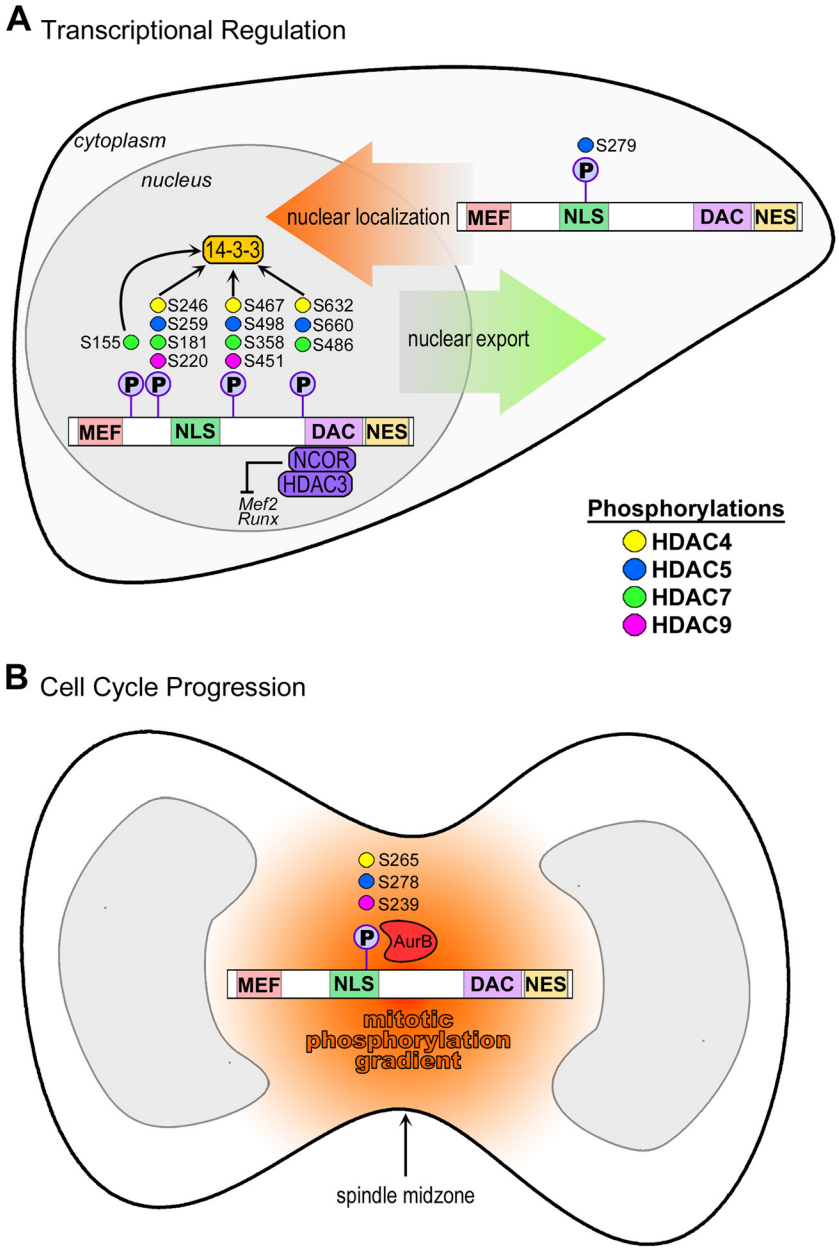

FIG. 3. **Phosphorylations and functions of class IIa HDACs are spatially and temporally regulated.** *A*, Nuclear HDACs repress transcription of target genes through associations with corepressor complexes. Phosphorylation of HDACs in the nucleus promotes binding of 14–3-3 chaperone proteins and nuclear export of all class IIa HDACs, whereas NLS phosphorylation promotes nuclear accumulation of HDAC5. *B*, In dividing cells, HDACs 4, 5, and 9 localize to the midzone within a mitotic midzone phosphorylation gradient and are phosphorylated by Aurora B within NLS.

this interaction (35). 14–3-3 association restricts the binding of HDAC4 to importin-α, indicating that inhibition of HDAC nuclear import likely occurs via occlusion of the NLS (35). A similar regulatory mechanism has been reported for other proteins, including Cdc25 in *Xenopus* XTC cells and ATXN1 in neurons (21, 124).

The phosphorylation sites important for nuclear export represent convergence points for multiple signaling pathways, most notably the CaMK superfamily, PKC/PKD, and MARK 1 and 2 (84). Ca^{2+}-mediated activation of the CaMK pathway promotes nuclear export of HDAC5 by stimulating phosphorylation of 14–3-3 binding sites (36). This change in localization triggers the dissociation of the MEF2/HDAC complex and subsequent activation of MEF2 transcriptional targets (36, 58, 125), a process observed to be essential for proper cardiac function (92, 126) (discussed in "The Post-translational Regulation of HDACs is Critically Linked to Human Disease"). In agreement, overexpression of HDAC4, HDAC5, and HDAC9 suppresses MEF2 activity in cardiomyocytes (16).

Similarly, phosphorylation of HDAC7 at its 14–3-3 binding sites by PKD promotes dissociation of HDAC7 from the transcriptional targets Nur77 (78, 80, 127), RUNX2 (82), and RCAN2 (81). Moreover, retention of HDAC7 in the nucleus results in repression of the matrix metalloproteinase MTI-matrix metalloproteinase (MTI-MMP) and MMP10, which are important for microvessel sprouting in angiogenesis (79). In addition to PKD signaling, the MARK kinases C-TAK1 and EMK also serve to regulate HDAC4-, HDAC5-, and HDAC7-

mediated repression of Nur77 and c-Jun (84). Interestingly, in HDAC7, the initial phosphorylation of one 14–3-3 binding site promotes the subsequent phosphorylation of the other chaperone binding sites (84), suggesting the cooperative regulation of PTMs within HDACs.

In addition to the PTMs flanking the NLS region, phosphorylation within the NLS at S279 is also critical for nuclear localization of HDAC5. Specifically, phosphorylation was shown to be important for efficient nuclear import in HEK293 and U2OS cells (28), whereas found to prevent PKD/CaMK-induced nuclear export in Cos7 cells (73).

Thus far, understanding of the PTM-induced changes in HDAC localization have been limited to the modification of the NLS region and 14–3-3 binding sites; however, recent studies have suggested that other PTMs can also trigger changes in HDAC5 protein association with important transcriptional regulators. Specifically, S611 mutation led to changes in the association of HDAC5 with the transcription factor MEF2D (12). Therefore, future studies investigating additional PTMs could shed light on their impact on HDAC localizations and interactions within different regions of the same subcellular compartments (*e.g.* subnuclear). Furthermore, it is worth noting that the aforementioned HDAC-mediated mechanisms are restricted to the effects of the presence or absence of HDACs on their nuclear roles. To date, the cytoplasmic functions of class IIa HDACs remain insufficiently explored, and are likely to be relevant in numerous cellular processes.

Cell Cycle-dependent Regulation of HDACs—As transcription is carefully regulated throughout cell cycle progression, it is expected that HDAC and histone acetyltransferases functions are also dynamic during different stages of the cell cycle. Along with changes in chromosome condensation status, histone acetylation levels have been shown to decrease early in mitosis, reaching their lowest levels during metaphase and anaphase, and rising at the late telophase/interphase transition (128). Additionally, global inhibition of HDACs using small molecules has been shown to inhibit mitotic progression by inducing G2-phase checkpoint response (129, 130). HDAC inhibition by Trichostatin A results in spindle and kinetochore attachment defects in mitotic cell populations, indicating that HDAC-containing complexes are important in properly maintaining mitotic structures (131). Additional HDAC inhibitors have been shown to promote cell cycle arrest at G0/G1 and at G2/M, and to be associated with increased apoptosis (132, 133). The acetylation status of spindle components has been proposed to be important for proper mitotic progression (131), and the class IIb enzyme HDAC6 is directly responsible for deacetylation of tubulin (17).

Changes in the distribution of HDAC4, HDAC5, and HDAC7 have been observed during mitosis, including the exclusion of HDAC4 from condensed chromatin regions (128). Moreover, HDAC5 was shown to localize to the spindle midzone during mitosis and to the midbody during cytokinesis, where it interacts with the mitotic kinase Aurora B and is phosphorylated at S278 within the NLS (47) (Fig. 3*B*). Phosphorylation of this site is conserved in HDAC4 and HDAC9, suggesting a shared point of regulation; however, in HDAC7 this NLS serine residue is replaced by lysine, indicating divergent regulation of HDAC7 from the rest of the class IIa family (47). Although phosphorylation of S278 coincides with HDAC localization to the mitotic midzone, whether S278 phosphorylation determines this redistribution, or whether the presence of HDAC5 at the midzone promotes its phosphorylation remains to be explored. Consistent with the midzone sequestration of HDAC5 during mitosis, interactions of HDAC5 with members of the NCoR complex were significantly diminished in a G2/M-arrested population of HEK293 cells, with a concomitant decrease in *in vitro* deacetylation activity (47). It is clear that histone acetylation profiles are dynamic during cell cycle progression, contributing to the regulation of gene expression during mitosis; therefore, further investigation of the mechanisms governing HDAC and histone acetyltransferase localization and activity during specific cell cycle stages will be an important resource for gaining a better understanding of transcriptional control throughout the cell cycle.

The Post-translational Regulation of HDACs is Critically Linked to Human Disease—Although class I HDACs have previously stirred significant interest for their roles in human disease progression and their therapeutic potential, in particular in cancers, studies in recent years have established unique functions for class IIa HDACs in different tissues and organs that can critically impact the development of disease. Given the extensive PTMs and localization-dependent functions of class IIa HDACs, and their intricate regulation by numerous signaling pathways, it is not surprising that these enzymes impact a wide range of cellular processes relevant to human health. Class IIa HDACs are expressed abundantly in the heart, brain, and musculoskeletal tissues, and known to regulate physiological processes in a tissue-specific manner (Fig. 4 and supplemental Table S1). The misregulation of class IIa HDAC levels, PTMs, localization, and transcriptional regulatory functions have been critically linked to significant human diseases, including cardiac, neurodegenerative, and musculoskeletal disorders. As such, these HDACs have emerged as potential novel targets for therapeutic interventions. This section presents an overview of the regulation and functions of class IIa HDACs in the context of human disease, as specifically connected to their PTM status.

Cardiovascular System and Cardiac Hypertrophy—One of the best established roles of class IIa HDACs in human disease is their impact on the cardiovascular system, in particular on the development of cardiac hypertrophy. The constant response of heart tissue to physiological and pathological stimuli can lead to increased cardiac cell size, also known as cardiac hypertrophy. Hypertrophy strengthens the cardiac wall in response to increased stress arising from hypertension, cardiac injury, valve disease, and myocardial infarction (134, 135); however, prolonged hypertrophy can lead to car-

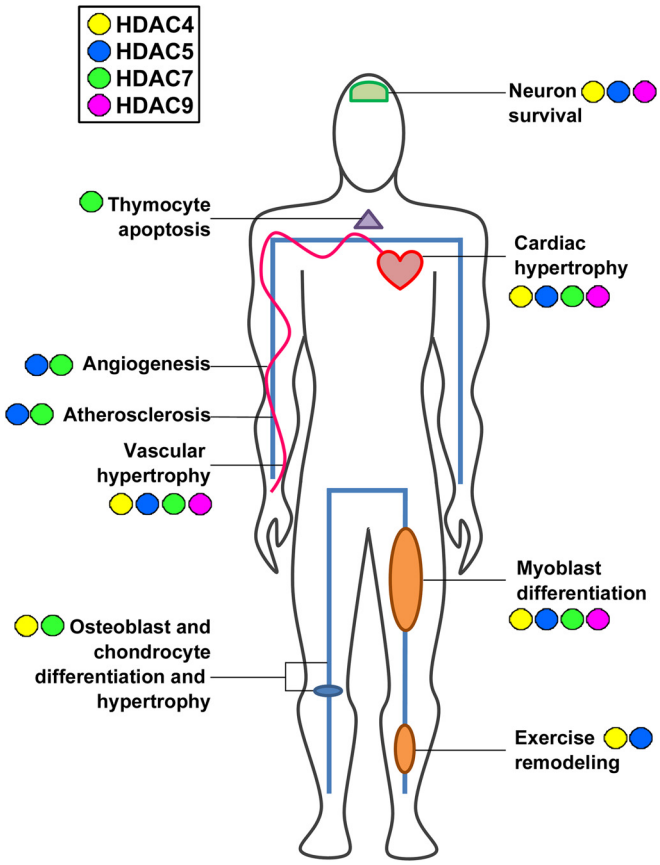

FIG. 4. **Class IIa HDACs have broad impact on the development of human disease.** HDAC4, 5, 7, and 9 have shared roles in cardiac development and muscle differentiation; however, specific roles for individual HDACs have been reported in certain organ systems and pathologies.

diomyopathy, fibrosis, and heart failure (136). Cardiac remodeling results from the activation of the "fetal gene program" that mediates the expression of proteins involved in muscle contraction and calcium absorption (137). In the absence of injury, this program is repressed by nuclear MEF2-interacting HDACs (138, 139). Hypertrophic stimuli activate a cardiac kinase that relieves HDAC-mediated repression thorough phosphorylation-dependent nuclear export (16). HDAC mutants lacking phosphorylation sites were refractory to hypertrophic stimuli, whereas HDAC9 knock-out mice displayed enhanced sensitivity to hypertrophic stimuli (16). Aberrant calcium signaling has also been implicated in the pathological remodeling of the heart in response to stress signaling (140) and the downstream phosphorylation of HDAC4 via CaMKI and CaMKII (40). Although some level of redundancy exists with regard to enzymes targeting multiple sites for phosphorylation (Fig. 1), several kinases only modify specific HDACs and/or sites. For example, CaMKIIδ-null mice display reduced kinase activity toward HDAC4, whereas HDAC5 was unaffected (41, 141). Additional specificity is conferred via the alternate splicing of CaMKIIδ, which yields localization-dependent phosphorylation of HDAC4 by a nuclear δB isoform activated by phenylephrine, and a cytoplasmic δC isoform activated by caffeine (27). Both isoforms can phosphorylate HDAC4 and induce similar MEF2 gene expression, and CaMKIIδC can also phosphorylate calcium regulatory proteins to modulate Ca^{2+} levels (42). Several other HDAC-modifying enzymes have been shown to induce cardiac hypertrophy via transmission of calcium-independent signals. PKD, a downstream effector of PKC signaling, can directly phosphorylate HDAC5 to stimulate its nuclear export (64, 142). Cardiac A-kinase anchoring proteins serve as scaffolds for PKA- and PKC-dependent activation of PKD1, thus facilitating nuclear export of HDAC5 (66). Export of HDAC5, and subsequent MEF2 activation, can also be triggered by nuclear GRK5 under hypertensive conditions, leading to ventricular hypertrophy (124, 143). Independently of blood pressure, high salt intake has also been known to be associated with left ventricle hypertrophy (128, 144). More recently, this mechanism was shown to be phosphorylation-dependent, as increased sodium levels activate the critical cardiac kinase SIK1, leading to HDAC5 phosphorylation and enhanced MEF2 and NFAT transcriptional activity (72). Interestingly, a recent study examined the mechanisms through which clinically relevant neurohormonal stimuli regulate HDAC phosphorylation in adult cardiomyocytes (62). Although PKD was found to be a critical mediator of endothelin-1-induced phosphorylation and nuclear export of HDAC5, β1-adrenergic receptor (β1-AR) also stimulated nuclear export and MEF2 activation through a phosphorylation-independent mechanism (62). Therefore, more research is needed to understand how these multiple pathways are integrated to achieve a common end point, such as fetal gene program activation.

Mechanisms that act to inhibit cardiac hypertrophy through nuclear retention of HDACs and repression of MEF2 activity further contribute to the challenge of fully understanding the regulation of HDACs during cardiac disease (supplemental Table S1). Such mechanisms are of particular interest as they can potentially be harnessed for therapeutic benefit. Phenylephrine signaling was shown to promote the formation of disulfide bonds between cysteine 667 and 669 of HDAC4 and nuclear export (54). Importantly, reduction of these disulfide bonds by thioredoxin 1 has the potential to limit cardiac hypertrophy (54). Specific phosphorylations are also capable of limiting HDAC nuclear export. PKA-mediated phosphorylation of HDAC5 at S279 impairs 14–3-3 binding, ultimately leading to nuclear retention, repression of MEF2-dependent transcription, and inhibition of cardiac hypertrophy (73). Activation of PKA by β1-AR also promotes HDAC5 nuclear accumulation, most likely through a PP2A-dependent mechanism (145). Another cardioprotective function of PKA involves the proteolytic cleavage of HDAC4, which releases an N-terminal fragment that translocates to the nucleus and represses MEF2 (57). Also inhibiting nuclear export is the direct binding

of the transcription factor YY1 to HDAC5 that prevents its phosphorylation (146).

Vascularization—Like cardiac muscle cells, vascular smooth muscle cells can also undergo pathological remodeling leading to hypertrophy via similar molecular mechanisms. Class IIa HDAC PTMs are also modulated under conditions of vascular hypertrophy. Phosphorylation of HDAC4 and HDAC5 in vascular smooth muscle cells is modulated by stimulation of PKD1 (65) and CaMKII (147). Formation of new blood vessels (angiogenesis) involves recruitment of both vascular smooth muscle cells and mature endothelial cells 126). During angiogenesis, VEGF induces phosphorylation of 14–3-3 binding sites and nuclear export of HDAC5 (60) and HDAC7 (79, 81). These phosphorylation events are mediated by PKD signaling, and result in the expression of VEGF-responsive genes (supplemental Table S1) that promote cell proliferation and migration (81), tube formation, and microvessel sprouting (60, 79). HDAC5 phosphorylation and localization are similarly regulated by CaMK during atherosclerosis, triggering enhanced expression of Kruppel-like factor 2 and endothelial nitric oxide synthase in endothelial cells (148). It has also been reported that nitric oxide, a product of endothelial nitric oxide synthase, induces PP2A-dependent dephosphorylation of HDAC4 (50). Lastly, PKD phosphorylation of HDAC5 has been shown to regulate erythropoiesis (149), suggesting that HDACs have roles in the development of both cardiovascular structures and blood cells.

Musculoskeletal System—Phosphorylation is also responsible for controlling the ability of class IIa HDACs to modulate gene programs associated with muscle and bone development, implicating them in diseases of the musculoskeletal system, including myopathies and osteoarthritis.

Muscle Development—Myogenesis encompasses the conversion of myoblasts into differentiated myotubes and involves the activation of hundreds of muscle-specific genes and concomitant repression of cell proliferation genes (14). In undifferentiated cells, MEF2 transcription factors are repressed when in a complex with HDAC4 or HDAC5 (133), which undergo nuclear export upon phosphorylation of 14–3-3 binding sites by GIT1 and CaMKs (27, 36). Although class IIa HDACs have a characteristic dual-compartment localization, individual HDACs exhibit different localization patterns upon myoblast differentiation. The pool of HDAC4 sequestered in the cytoplasm returns to the nucleus following myoblast fusion; however, CaMKIV can inhibit HDAC4 nuclear relocalization (38). Interestingly, HDAC7 is exclusively cytoplasmic in differentiated myotubes (150). The HDAC9 splice variant MITR inhibits the myogenic program, and is also subject to CaMK and Mirk/dyrk1B-dependent phosphorylation-dependent subcellular localization (48, 90). Mirk targets residues within the NLS domains of HDAC5 and MITR, rather than the 14–3-3 binding sites targeted by CaMKs (Fig. 1).

Bone Development—Skeletal biology, including the differentiation of osteoblasts and chondrocytes, is regulated by bone morphogenic proteins (BMPs) that establish bone formation and growth (151, 152). BMP2 induces nuclear export of HDAC7, but not of HDAC5, via PKD-mediated signal-dependent phosphorylation (82). This process relieves the HDAC7-mediated repression of Runx2, the master transcriptional regulator of the skeletal system (153). Differentiation of proliferating chondrocytes into prehypertrophic chondrocytes is also induced by CaMKIV-mediated nuclear exclusion of HDAC4 and elevated Runx2 activity (46), whereas HDAC4-null mice exhibit premature bone ossification (154), implicating these regulatory mechanisms in the development of osteoarthritis. This maturation process can be reversed by expression of the parathyroid hormone-related peptide, which represses Runx2 via PP2A-dependent dephosphorylation of HDAC4 (52), suggesting that modulation of HDAC phosphorylation may represent a promising therapeutic for osteoarthritis and bone calcification disorders.

The Nervous System and Neurodegenerative Diseases—The cytoplasmic localization of class IIa HDACs in neurons allows MEF2 and CREB to express numerous genes involved in neuronal differentiation (155). Neuronal activity itself specifies HDAC subcellular localization; spontaneous synaptic activity is sufficient to induce nuclear export of HDAC4, whereas export of HDAC5 requires CaMK inhibition and calcium flux (156). Similarly, phosphorylation-dependent HDAC9 translocation occurs following spontaneous firing in cortical neurons (91), and subsequent expression of c-fos promotes dendritic growth. Knowledge of the cytoplasmic functions of these HDACs is currently limited; however, multiple studies indicate that the roles and regulatory mechanisms of HDACs in the cytoplasm are highly relevant to understanding their contribution to human disease progression.

In the cytoplasm of peripheral neurons, HDAC5 deacetylates tubulin, promoting neuro-regenerative growth (157). HDAC5 also regulates axon regeneration when activated by PKC (157), further indicating that phosphorylation is an important determinant of HDAC function during neuronal development and in the presence of neuronal injury. Cocaine treatment induces the nuclear export of HDAC5 and activation of MEF2C via SIK1-dependent phosphorylation (158). Interestingly, a separate study has demonstrated that cocaine can alternatively induce nuclear accumulation of HDAC5 through cAMP signaling and PP2A-mediated dephosphorylation of S279 (74). Thus, it is possible that multiple signaling pathways may direct differentially phosphorylated HDAC species to the relevant subcellular compartments under such stress conditions.

HDAC4 has a substantial cytoplasmic localization in neurons (159). Although the nuclear accumulation of HDAC4 because of increased PP2A activity is associated with ataxia telangiectasia (53), the cytoplasmic functions of HDAC4 may also be involved in neurodegenerative diseases. Huntington's disease is caused by the mis-folding of the huntingtin protein, and its pathology is associated with the formation of both

nuclear and cytoplasmic aggregates (160). HDAC4 has emerged as a possible promising target for the treatment of Huntington's disease because of its association with the huntingtin protein and its ability to influence the formation of cytoplasmic aggregates (21). As HDAC4 localization is determined by its phosphorylation status, these signaling pathways could be exploited to promote the nuclear retention of HDAC4 in order to limit the formation of cytoplasmic aggregates associated with Huntington's disease pathogenesis. This observation is especially noteworthy as the cytoplasmic roles of class IIa HDACs remain poorly characterized, and increased understanding of such roles will likely be of significant clinical relevance.

Emerging Roles for Class IIa HDACs in Cancer and Diabetes—Although the PTM status of class IIa HDACs has been established to modulate their functions in cardiovascular and neurodegenerative disease, recent studies indicate that their roles in human disease may be far reaching.

It is well-established that HDACs have roles in cancer development. The broad HDAC inhibitor Vorinostat is currently approved for treatment of T-cell lymphomas, indicating that HDACs represent important targets for limiting cancer progression. Thus far, class I HDACs have been the major focus of cancer studies, as their loss of function is linked to cancer development (132). However, class II HDACs may also represent important regulators of tumorigenesis. In gastric cancer cells, PKD2 accumulates in the nucleus and induces phosphorylation of HDAC7, ultimately resulting in HDAC7 nuclear export and expression of downstream targets including Nur77 (83). Conversely, HDAC4 localizes to the nucleus in cancerous colon cells, where it interacts with Sp1 and represses transcription of p21, promoting cancer cell growth (161). Additional investigation of class IIa HDAC localizations, post-translational modifications, and functions in multiple cancer types will provide important insight into their roles in tumor development.

The pathogenesis of diabetes is also linked to HDAC function and involves resistance to insulin in tissues such as skeletal muscle. The fasting hormone glucagon induces dephosphorylation and nuclear retention of HDAC4, HDAC5, and HDAC7 (20). Surprisingly, however, the expression of gluconeogenic genes, such as the catalytic subunit of G6Pase, requires class IIa HDACs to deacetylate and activate FOXO transcription factors (20), indicating that HDACs are able to regulate the production of hormones that can control blood glucose levels. Additionally, HDAC5 modulates the expression of GLUT4, a key regulator of insulin resistance, via phosphorylation-dependent nuclear export and derepression (69). Interestingly, HDAC5 phosphorylation levels have been shown to be elevated during aerobic exercise (162), which could indicate that HDACs respond to change in blood oxygen levels. The broader family of human deacetylases is known to respond to changes in cellular metabolic environments. It is well established that the class III deacetylase (SIRT) activities are regulated by NAD^+, and their enzymatic activities can be modulated by glucose and glutamine availability (163–166). Moreover, acetyl-CoA and other CoA derivatives, along with NADPH, have been shown to allosterically activate class I enzymes, HDAC1 and HDAC2 (167). Additional metabolic regulators of HDACs include short chain fatty acids (*e.g.* butyrate), which formed the basis for the design of chemotherapeutic treatments (168). Increased abundance of short chain fatty acids and butyrate have been proposed to contribute to the cardioprotective and antidiabetic roles of dietary whole grains (169). It is possible that alterations in butyrate concentrations could contribute to modulating HDAC activities in diabetic patients.

Perspectives—Class IIa HDACs are comprehensively controlled by numerous signaling pathways and enzymes that mediate their post-translational modification (Fig. 1). These modifications are found in multiple protein domains and areas of disordered structure (Fig. 2), and not only alter HDAC subcellular localization, but fundamentally impact the ability of HDACs to repress/activate targets. Accumulating evidence indicates that site-specific PTMs control distinct spatial and temporal HDAC regulatory mechanisms, such as via mediating protein interactions, dynamic localization, and cell cycle-dependent effects. Nuclear export of class IIa HDACs has been generally investigated in the context of its ability to regulate transcription. However, the cytoplasmic functions of HDACs are now starting to be more widely explored, particularly with respect to their impacts on human diseases, including neurodegenerative disorders, cardiac disease, and virus-induced pathologies.

The HDAC regulatory network is highly complex, and is responsive to both tissue context and environmental cues. Proteomic studies and advances in mass spectrometry-based methods provide valuable tools for further expanding the identification of PTMs within these different biological contexts. Characterizing these modifications, their regulation by specific enzymes, and their possible coordinated functions in different tissues and under physiological conditions will be critical for gaining a deeper understanding of organ- and disease-specific HDAC functions (Fig. 4). Furthermore, multidisciplinary approaches integrating molecular, clinical, and mass spectrometry-based methods, promise to generate a systems-wide understanding of the dynamic regulation of HDACs. Given the critical links starting to be established between HDAC PTM status and human diseases, the quantification of site-specific PTMs by targeted mass spectrometry may provide accurate and sensitive detection of disease biomarkers in clinical samples.

HDACs have been exploited for their therapeutic benefit given their involvement in conditions such as cardiac and vascular hypertrophy, atherosclerosis, cancer, and diabetes. With several HDAC inhibitors in phase I and II clinical trials, and the development of small molecule inhibitors that block the MEF2 interaction, it is only a matter of time before the full

potential of HDAC regulatory mechanisms are realized and used to treat human diseases and disorders. As numerous small molecules can simultaneously inhibit multiple HDACs, the use of such drugs can alter numerous gene programs and lead to cytotoxicity. Gaining a better understanding of HDAC regulation promises to provide targets with increased specificity that can selectively modulate a single HDAC or a given context-dependent function. Such targets may include kinases/phosphatases or other enzymes regulating a certain PTM site or factors influencing the formation of HDAC protein interactions.

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[S] This article contains supplemental Table S1.

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