Aurora B-dependent Regulation of Class IIa Histone Deacetylases by Mitotic Nuclear Localization Signal Phosphorylation*

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Class IIa histone deacetylases (HDACs 4/5/7/9) are transcriptional regulators with critical roles in cardiac disease and cancer. HDAC inhibitors are promising anticancer agents, and although they are known to disrupt mitotic progression, the underlying mechanisms of mitotic regulation by HDACs are not fully understood. Here we provide the first identification of histone deacetylases as substrates of Aurora B kinase (AurB). Our study identifies class IIa HDACs as a novel family of AurB targets and provides the first evidence that HDACs are temporally and spatially regulated by phosphorylation during the cell cycle. We define the precise site of AurB-mediated phosphorylation as a conserved serine within the nuclear localization signals of HDAC4, HDAC5, and HDAC9 at Ser265, Ser278, and Ser242, respectively. We establish that AurB interacts with these HDACs in vivo, and that this association increases upon disruption of 14-3-3 binding. We observe colocalization of endogenous, phosphorylated HDACs with AurB at the mitotic midzone in late anaphase and the midbody during cytokinesis, complemented by a reduction in HDAC interactions with components of the nuclear corepressor complex. We propose that AurB-dependent phosphorylation of HDACs induces sequestration within a phosphorylation gradient at the midzone, maintaining separation from re-forming nuclei and contributing to transcriptional control. Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.021030, 1220–1229, 2012.

Class IIa histone deacetylases (HDACs1 4, 5, 7 and 9) are prominent epigenetic regulators of gene expression with critical roles in cardiac disease (1), epigenetic response to drug stimulus (2), cancer (3), and viral infection (4). Distinct from transcriptional regulators that directly alter DNA sequences, gene expression is indirectly mediated by class IIa HDACs through a mechanism of nucleo-cytoplasmic shuttling (5). Alterations in HDAC localizations are critically linked to human disease. Increased nuclear export of class IIa HDACs was shown in the failing human heart, contributing to selective induction of gene programs associated with dilated cardiomyopathy (6, 7). Furthermore, HDAC5 localization and expression levels were reported to change dramatically following HIV-1 infection (8), though the pathways contributing to this phenomenon are not well understood. Thus, exploring the mechanisms governing HDAC localizations and functions is crucial to uncovering their roles in disease progression.

Regulation of the nucleo-cytoplasmic shuttling of class IIa HDACs involves dynamic, site-specific phosphorylation (9–14). In HDAC5, two phosphorylations flanking the nuclear localization signal (NLS) promote binding of 14-3-3 chaperone proteins and export of HDAC5 to the cytoplasm, whereas their de-phosphorylation facilitates nuclear import (5, 12). Recently, our lab reported numerous additional HDAC5 phosphorylation sites, several of which are present within the NLS, nuclear export signal, and deacetylation domain (15). In particular, two adjacent phosphorylations within the NLS (Ser278 and Ser279) were of significant interest. We showed that phosphorylation at Ser279 is necessary for efficient nuclear import of HDAC5 in kidney cells (HEK293) and osteosarcoma cells (U2OS) (15). The kinases responsible for Ser279 phosphorylation have recently been identified to be PKA in Cos-7 cells (16) and Cdk5 in neurons (17). In contrast, the role of the neighboring NLS phosphorylation at Ser278 remains unexplored. Given the wealth of phosphorylation sites within HDAC5 and conservation of multiple residues among class IIa HDACs, it is likely that these enzymes are spatially and temporally regulated by a broader spectrum of kinases than has previously been documented.

The concerted actions of HDACs and histone acetyltransferases are proposed to be dynamically regulated during cell cycle progression to modulate histone acetylation during mitosis. Specifically, global inhibition of HDAC activity interferes with mitotic progression (18, 19), triggering G2-phase checkpoint response (20, 21), chromosomal instability (18), and
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defects in kinetochore assembly (22). Moreover, the class I deacetylase HDAC3, a known interacting partner of class IIa HDACs, is linked to G1/S checkpoint maintenance and is reported to localize to the mitotic spindle and modulate spindle assembly and kinetochore attachment (23). Yet, the contribution of class IIa HDACs to mitotic progression is unknown.

Here, we determine that Aurora B (AurB), an essential mitotic kinase, phosphorylates class IIa HDACs in vitro and in vivo at a single conserved serine within the NLS. We also demonstrate that AurB interacts with class IIa HDACs, and that this interaction is augmented in NLS-phosphorylated HDACs. We show that AurB-dependent NLS phosphorylation of HDACs is enriched in mitosis, and that the phosphorylated HDACs are sequestered at the midbody during mitosis, with a parallel loss in interactions with members of the nuclear corepressor complex (NCoR).

EXPERIMENTAL PROCEDURES

Antibodies—The antibodies used in these experiments for immunofluorescence studies were an in-house developed rabbit polyclonal antibody against GFP, as described (24), monoclonal anti-GFP (Roche), MAb414 (Covance, gift from J. Glavy, Stevens Institute), Aurora B (Abcam, Cambridge, MA), GAPDH (Abcam), 14-3-3 (T-16, Santa Cruz, CA), H3pS10 (Millipore), Tubulin (Roche), NCoR1 (Fisher Scientific), TBL1X (Santa Cruz Biotechnology, Santa Cruz, CA), TBL1XR1 (Santa Cruz Biotechnology), and HDAC3 (Santa Cruz Biotechnology). The rabbit polyclonal phosphospecific antibody against HDAC5-pS278 was generated for this study using a synthetic peptide containing the phosphorylated Ser278 residue of HDAC5 (QKVAERR-pS-p). The rabbit polyclonal phosphospecific antibody against HDAC5-pS278 was generated for this study using a synthetic peptide containing the phosphorylated Ser278 residue of HDAC5 (QKVAERR-pS-p).

Cell Line Construction—For these experiments, we used previously described U2OS or HEK293 cell lines stably expressing EGFP-FLAG-tagged wild-type HDAC5 or phosphomutant HDAC5 (S278A, S278/279A, and S259/498A), which we constructed using the PhoenixTm retrovirus expression system (Orbigen, San Diego, CA) (15). CEM T cell lines stably expressing EGFP-FLAG-tagged HDAC4, HDAC5, and HDAC9 were generated for this study, using the procedure described (19). HDAC4 and HDAC9 plasmids were a kind gift of E. Seto (25). EGFP was obtained from pEGFP-N1 (Clontech, Mountain View, CA). Cells stably expressing tagged protein were selected with 300 mg/ml G418 (EMD, Gibbstown, NJ) and sorted by FACs (Vantage S.E. with TurboSort II, Becton Dickinson, Franklin Lakes, NJ) as previously described (26). HDAC5 phosphomutant cell lines were generated by site-directed mutagenesis of the existing tagged-HDAC5 constructs. The list of primers used for this study is provided as supplementary material, supplemental Table S1.

Immunofluorescence Purification of HDACs—EGFP-tagged HDACs (wild-type or phosphomutants) and control EGFP were isolated by immunofluorescence purification via the EGFP tag on magnetic beads conjugated with in-house generated anti-GFP, as previously described (24, 26). For mass spectrometry analyses of immunopurified HDACs, 0.5 and 2 g of cells were used as starting material to account for the differential expression levels of the tagged HDACs (0.5 g cells for HDAC5, 1.5 g for HDAC4, and 2 g for HDAC9). The starting material was 0.2 g of cells for IP-Western experiments. Briefly, HEK293 and/or CEM T cells expressing tagged-HDACs or EGFP were harvested, frozen, and cryogenically lysed using a Retch MM301 Mixer Mill (Retch, Newtown, PA), as described (15). For isolation of EGFP-tagged HDACs, the resulting frozen cell powder was homogenized in lysis buffers that were individually optimized for effective isolations of each HDAC. HDAC5 cells were suspended in 20 mM HEPES-KOH, pH 7.4, 0.1 M potassium acetate, 2 mM MgCl2, 0.1% Tween-20, 1 μM ZnCl2, 1 μM CaCl2, 0.5% Triton X-100, 250 mM NaCl, 4 μg/ml DNase I, 1/100 (v/v) protease, and phosphatase inhibitor cocktails (Sigma). HDAC4 isolations were performed with a lysis buffer containing 20 mM HEPES-KOH, pH 7.4, 0.1 M potassium acetate, 2 mM MgCl2, 0.1% Tween-20, 1 μM ZnCl2, 1 μM CaCl2, 1.0% Triton X-100, 0.5% sodium deoxycholate, 200 mM NaCl, 4 μg/ml DNase I, 1/100 (v/v) protease, and phosphatase inhibitor cocktails (Sigma). HDAC9 isolations were performed using 20 mM HEPES-KOH, pH 7.4, 0.1 M potassium acetate, 2 mM MgCl2, 0.1% Tween-20, 1 μM ZnCl2, 1 μM CaCl2, 0.3% Triton X-100, 150 mM NaCl, 4 μg/ml DNase I, 1/100 (v/v) protease and phosphatase inhibitor cocktails. All isolations were subjected to homogenization by Polytron (Kinematica), if the initial cell powder used was equal or greater than 0.5 g, or by vortexing if the initial cell powder used was equal to 0.2 g, followed by centrifugation at 8000 × g for 10 min at 4 °C. Affinity isolations were performed by incubation with conjugated magnetic beads (M-270 epoxy beads, Invitrogen) for 1 h (HDAC4 and HDAC5 isolations) or 2.5 h (HDAC9 isolations) at 4 °C, as described (27). Immunoisolates were eluted in 30–50 μl 1X LDS sample buffer for 10 min at 70 °C. LDS-eluted complexes were processed immediately via in-gel or in-solution digestion for complementary mass spectrometry analyses, as described (28).

Mass Spectrometry-based Proteomic and Data Analysis—Class IIA HDAC immunoisolates were either digested in-solution or in-gel, as previously described (28). Briefly, reduced and alkylated proteins were digested in-solution with 10 μl of trypsin (5 ng/μl) in 100 mM ammonium bicarbonate (ABC) using Vivaco 500 cristifugal filters (10 KdA MWCO; Sartorius Stedim Biotech, Goettingen, Germany), as described (29). For in-gel digestion, reduced and alkylated proteins were resolved by SDS-PAGE for ~3 cm and digested with either 10 μl of trypsin (12.5 ng/μl) in 50 mM ABC for 6 h at 37 °C. Peptides were separated by vacuum centrifugation to ~10 μl and analyzed by nLC-MS/MS on a Dionex Ultimate 3000 RSLC coupled directly to an LTQ-Orbitrap Velos ETD mass spectrometer (ThermoFisher Scientific, San Jose, CA), as previously described (29). Peptides were either desalted online (trap column; Magic C18 AQ, 3 μm, 100 μm × 2.5 cm, Michrom Bioreources, Inc.) or offline by StageTips (30). Peptides were separated by reverse phase chromatography (Acclaim PepMap RSLC, 1.8 μm, 75 μm × 25 cm) at a flow rate of 250 nl/min using a 90 or 180 min gradient for in-gel or in-solution digested samples, respectively.

The mass spectrometer was operated in data-dependent acquisition mode with FT preview scan disabled and predictive AGC and dynamic exclusion enabled (repeat count: 1, exclusion duration: 70 s). A single acquisition cycle comprised a single full-scan mass spectrum (m/z ~ 350–1700) in the Orbitrap (f = 30,000 at m/z 400), followed by collision-induced dissociation (CID) fragmentation of the top 20 most intense precursor ions (min signal = 1E3) in the dual-pressure linear ion trap. FT full scan MS and IT MS2 target values were 1E6 and 1E5, and maximum injection times were set at 300 and 100 ms, respectively. CID fragmentation was performed at an isolation width of 2.0 Th, normalized collision energy of 30, and activation time of 10 ms.

MS/MS spectra were extracted, filtered, and searched by Proteome Discoverer/SEQUEST (v1.3 Thermofisher Scientific) against a human protein sequence database (Uniprot-SwissProt, 2010-11) ap-
performed (1) in the absence of ATP and (2) with addition of 1 M ATP and 50 ng of recombinant human AurB kinase (Cell Signaling Technology, Boston, MA) and staurosporine, a general serine/threonine kinase inhibitor. Reactions incubated at room temperature for 30 min. Control assays were performed using PeptideProphet (31) in Scaffold, and Percolator (32) in Proteome Discoverer. Probability filters were selected empirically to reduce the global peptide false discovery rate to less than 1%. Phosphorylation site localization was confirmed by manual inspection of MS/MS spectra and evaluation by the phosphoRS algorithm (Proteome Discoverer, v1.3). As previously described (15), Scaffold was used to facilitate label-free spectral counting analysis and assess the relative abundance changes of well-established class IIa HDAC interactions after G2/M arrest. A minimum of five total spectra in each biological replicate in at least one treatment condition (DMSO (n = 4), NOC (n = 5), HU (n = 3)) was required for relative quantification. To correct, for differences in immunoinosulated HDACs bait, prey protein spectral counts in the NOC and HU replicates were normalized to respective HDAC5-EGFP spectral counts in the DMSO controls. Spectrum count fold change was calculated as the ratio of averaged spectrum counts for individual proteins between treatment condition.

Protein and peptide identification tables are provided for Aurora B and selected HDAC5-interacting partners in Supplemental Tables S2 and S3, respectively.

**In Vitro Kinase Assay for HDAC Phosphorylation**—A synthetic peptide substrate corresponding to the NLS region, KAERRSSPLRRK, was used to determine the in vitro specificity of AurB-dependent phosphorylation. Kinase assays were performed in 20 mM Tris-HCl, pH 7.6, containing 10 mM MgCl₂, phosphatase inhibitors (1:100, Sigma), 2 mM dithiothreitol, and 2 μM NLS peptide substrate. Kinase reactions were initiated by addition of 200 μM ATP and 50 ng of recombinant human AurB kinase (Cell Signaling Technology, Boston, MA) and incubation at room temperature for 30 min. Control assays were performed (1) in the absence of ATP and (2) with addition of 1 μM staurosporine, a general serine/threonine kinase inhibitor. Reactions were terminated by addition of trifluoroacetic acid to 0.5%. The peptide substrate was desalted by StageTips (30), eluted in 100 μl of 50% acetonitrile containing 0.1% formic acid, and directly infused into an ESI LTQ-Orbitrap Velos mass spectrometer at 0.5 μl/min. Peptide sequence and phosphorylation sites were determined by tandem mass spectrometry using electron transfer dissociation (ETD) of the 4+ charge state ion and detection of fragment ions in the Orbitrap analyzer (resolution = 7500).

**Confocal Microscopy**—Wild-type HEK293 cells or HEK293 and U2OS cells stably expressing HDAC5-EGFP-FLAG were cultured on glass coverslips, pretreated with poly-d-Lys (Sigma, St. Louis, MO). After 24 h, cells were fixed with 2% paraformaldehyde, washed with 0.1 M glycine/0.2% PBS, permeabilized with 0.1% Triton X-100 in 0.2% PBS (PBST) for 15 min and blocked in 2% (v/v) bovine serum albumin, 0.2% (v/v) Tween-20 in 0.2% PBST at RT for 60 min. Incubation with primary antibody was performed at RT for 60 min in blocking buffer. The cells were then washed with PBST and incubated with either goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to Alexa 488, 568, or 633 (Invitrogen/Invitrogen). Cells were incubated with 1 μg/ml DAPI in PBS for 15 or 30 min and visualized by confocal microscopy on a Zeiss LSM510 (Zeiss, Dublin, CA) using 63× oil-immersion lens or 100× oil-immersion lens. Coverslips were mounted on slides with Aqua-Poly/Mount media (Polysciences).

**AurB Inhibition and Cell Cycle Arrest**—HEK293 cells expressing HDAC5-EGFP were seeded and cultured to 30% confluence, after which Hesperadin (Selleck Chemicals, Houston, TX) was added to the standard medium (Dulbecco’s modified Eagle’s medium (Invitrogen), 10% fetal bovine serum (Gemini MedSupply Partners, Atlanta, GA), 1% Pen/Strep (Gemini MedSupply Partners)) to final concentrations of 20, 40, or 60 nM. Hesperadin-treated and control (DMSO-treated) cells were cultured for 24 h then harvested via trypsinization and centrifugation. Whole cell lysates were prepared using the previously described lysis buffer and samples were analyzed by SDS-PAGE and Western blotting. HDAC4-EGFP and HDAC9-EGFP whole cell lysates were similarly prepared from CEM T suspension cells treated with Hesperadin (20/40/60 nM) in standard suspension media (Roswell Park Memorial Institute (Invitrogen), 10% fetal bovine serum, 1% Pen/Strep). Suspension cells were harvested by centrifugation and prepared for Western blot analysis. Blots were probed with custom anti-pS278 (YenZym), anti-H3pS10, and anti-GAPDH antibodies. Additional analyses of whole cell lysates prepared from HEK293 cells expressing HDAC5-EGFP cultured in the presence of Hesperadin (40 nM final concentration) were performed by Western blotting, as was previously described. Cell cycle arrest was induced in HEK293 cells expressing HDAC5-EGFP by treatment with nocodazole (Sigma) (G2/M) at a final concentration of 100 ng/ml for 20 h or with hydroxyurea (Sigma) (G1/S) at a final concentration of 4.0 mM for 40 h. siRNA inhibition of AurB was performed using the reverse transfection protocol with the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen/Invitrogen) and MISSION® esiRNA targeting human AURKB (Sigma-Aldrich), 3.4 μM final concentration.

**RESULTS AND DISCUSSION**

**HDAC5 Localizes to the Midbody During Cytokinesis**—The mitotic regulation of HDAC5 was investigated by first assessing its cell cycle-dependent localization in U2OS cell lines stably expressing functional EGFP-tagged HDAC5 (HDAC5-EGFP). As we previously reported, during interphase, HDAC5-EGFP exhibited both nuclear and cytoplasmic localizations, consistent with the shuttling mechanism of class IIa HDACs (12, 15). Interestingly, during mitosis and cytokinesis, HDAC5-EGFP had a partial localization at the spindle midzone and midbody (Fig. 1A).

The distinct midbody localization of HDAC5 led us to predict that AurB may phosphorylate HDAC5 in a cell cycle-dependent manner. Essential for progression through cytokinesis is the mitotic kinase Aurora B, which localizes at the spindle midzone and midbody of dividing cells (33). The spindle midzone contributes to determination of cytokinesis furrow placement and to localization of midzone-associated proteins, including members of the chromosome passenger complex and kinase (34). At the onset of mitosis, AurB localizes to centromeres, transitioning to the midzone during late anaphase (35, 36), promoting formation of an intracellular phosphorylation gradient that influences localization of phosphorylated protein variants (37). Reported AurB substrates include histone H3 (38–40) and the centromere-associated histone H3 variant CENP-A (41), whose phosphorylations are critical for chromosome segregation during mitosis (42). Recently, additional candidate AurB substrates were identified in an elegant large-scale proteomics study in HeLa cells (43).
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Aurora B Consensus: (R/K)_{1–3}–S–[S/T] (44) (Fig. 1A). Ser278 is conserved in multiple class IIa HDACs, as shown by sequence alignment of HDAC4, HDAC5, and HDAC9, indicative of a common point of regulation by AurB.

However, histone deacetylases have not yet been reported as AurB substrates, nor, to our knowledge, have they been shown to be dynamically regulated by phosphorylation during the cell cycle.

NLS Sites Are Phosphorylated In Vivo within Both HDAC4 and HDAC5—Interestingly, of the multiple phosphorylation sites that we previously reported for HDAC5 (15), a single site, Ser278 within the NLS, matched the AurB consensus sequence (Fig. 1A). Ser278 matches the AurB-consensus sequence (16). Using a synthetic peptide corresponding to the conserved NLS region that contains the two adjacent phosphorylation sites: (R/K)_{1–3}–S–[S/T] (44) (Fig. 1B). Ser278 is conserved in three of the four class IIa HDACs (HDAC4, HDAC5, and HDAC9), indicative of a common point of regulation by AurB.

Aurora B Associates with Immunoaffinity Purified Class IIa HDACs—To further confirm the association of AurB with the other class IIa HDACs, we generated cell lines stably expressing EGFP-tagged HDAC4 and HDAC9. Indeed, immunoaffinity purifications of HDAC4-EGFP and HDAC9-EGFP identified AurB as a co-isolating protein (Figs. 2B, 2C), whereas AurB was absent from EGFP control purifications, further supporting the functional connection between AurB and deacetylases. Copurification of AurB with class IIa HDACs containing the AurB-consensus sequence, in conjunction with localization of HDAC5 with AurB during late mitosis, provides further evidence that class IIa HDACs are common mitotic substrates of AurB.

Conserved Ser278 is a Substrate for Aurora B Phosphorylation In Vitro—Having demonstrated colocalization and interaction of AurB and HDAC5-EGFP, we next examined whether AurB could directly phosphorylate Ser278 in vitro. We generated a synthetic peptide corresponding to the conserved NLS region that contains the two adjacent phosphorylation sites: Ser278, the putative AurB site; and Ser279, the PKA-responsive site (16). Using an in vitro kinase assay and mass spectrometry analysis, we demonstrated that AurB can phosphorylate Ser278. This phosphorylation occurs in vivo within multiple class IIa HDACs. Our previous results identified Ser278 phosphorylation on HDAC5 in HEK293 cells (15). To determine whether the equivalent site in HDAC4, Ser265, is phosphorylated, we generated a cell line stably expressing EGFP-tagged HDAC4. Following immunoaffinity purification of HDAC4 and targeted MS/MS analysis, we demonstrated that Ser265 is indeed phosphorylated in CEM T cells (Fig. 1C). Moreover, the neighboring NLS site in HDAC4 (Ser266) was also phosphorylated, consistent with our previous observation of Ser279 phosphorylation in HDAC5 (15). The phosphorylations at Ser278 in HDAC5 and Ser265 in HDAC4 demonstrate conservation of this putative AurB site among multiple class IIa HDACs.

**Fig. 1.** HDAC5 localizes to the midbody during cytokinesis and NLS sites are phosphorylated in vivo within both HDAC4 and HDAC5. A, Localization of HDAC5-EGFP in U2OS cells undergoing cytokinesis; HDAC5 (green, EGFP), nuclear periphery (red, Mab414 against nuclear pore complex proteins), nucleus (blue, DAPI), 100× oil-immersion lens, 2× optical zoom. Scale bar = 50 μm. B, Sequence alignment of conserved HDAC NLS regions. The NLS (gray box) is conserved among class IIa HDACs, as shown by sequence alignment of HDAC5 (NP_005465.2), HDAC4 (NP_006028.1), HDAC7 (NP_001091885.1), and HDAC9 (NP_848512.1). Ser278 matches the consensus sequence AurB-phosphorylation (bold). C, HDAC4 is phosphorylated within the NLS at S266 and S267, the analogous sites to S278 and S279 in HDAC5, as demonstrated by CID MS/MS of immunoaffinity purified HDAC4-EGFP.
ylate the NLS peptide, as indicated by the detection of the phosphorylated NLS peptide (m/z = 444.7580, z = 4) (Fig. 3A). Furthermore, phosphorylation was abolished upon inhibition of AurB with staurosporine, a broad-acting serine/threonine kinase inhibitor. The unmodified NLS peptide (m/z = 424.766, z = 4) was observed in all conditions. To determine whether AurB-mediated phosphorylation occurred exclusively at Ser278, the phosphorylated NLS peptide was fragmented by electron transfer dissociation (ETD). The presence of prominent site-determining c7 and z8 fragment ions demonstrated conclusively that AurB phosphorylated Ser278, but not the adjacent Ser279 (Fig. 3B). Because the NLS peptide sequence used in the assay represents a highly conserved region of class IIa HDACs, this result further indicates that HDACs 4, 5, and 9 may all be targets for regulation by AurB kinase.

Class IIa HDACs are In Vivo Substrates of Aurora B—To assess whether this conserved phosphorylation is dependent on AurB in vivo, we generated custom phospho-specific antibodies against a synthetic peptide containing the phosphorylated Ser278 and its flanking regions (anti-pSer278). Antibody specificity toward pSer278 was evaluated by Western blotting of whole cell lysates prepared from cell lines expressing wild-type (WT) HDAC5-EGFP, Ser278Ala (S278A) and Ser278/279Ala (S278/279A) HDAC5-EGFP phosphomutants, and control EGFP-expressing (EGFP) HEK293 cells. Anti-pSer278 specifically recognized WT HDAC5-EGFP (Fig. 4A). Because mutation of Ser278 to alanine does not prevent phosphorylation at Ser279 (15) and immunoreactivity was eliminated in the Ser278Ala phosphomutant, we confirmed antibody specificity for Ser278 phosphorylation and not the adjacent Ser279 phosphorylation.

Using the anti-pSer278 antibody, we tested whether phosphorylation of Ser278 in HDAC5 is dependent upon AurB activity. To inhibit AurB expression in cell lines stably expressing HDAC5-EGFP, we transfected HEK293 cells with siRNA targeting human AURKB (AurB) and observed a significant decrease in Ser278 phosphorylation in whole cell lysates compared with cells transfected with non-targeting siRNA (Fig. 4B). Moreover, treatment of HDAC5-EGFP expressing cells with Hesperadin, a potent and selective inhibitor of AurB, caused a dose-dependent reduction in Ser278 phosphorylation as measured by Western blotting (Fig. 4C). Consistent with the presence of the same AurB consensus sequence in HDAC4 and HDAC9, we observed a similar reduction in pSer265 HDAC4-EGFP and pSer242 HDAC9-EGFP following inhibition of AurB in cell lines stably expressing the tagged HDACs (Figs. 4D, 4E). The known AurB target, Ser10 in histone H3, acted as a positive control. Because the endogenous class IIa family members have similar molecular weights and possess conserved NLS phosphorylations, use of stable EGFP-tagged HDAC-expressing cell lines allowed differentiation of class IIa HDACs by analyzing individual EGFP-tagged phosphorylated HDACs (~150 kDa). Overall, these results
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Aurora B-dependent Phosphorylation of HDACs is Spatially Regulated During Mitosis—Activation of AurB kinase at the midzone and the resulting changes in substrate phosphorylation are crucial regulators of late mitosis and cytokinesis (37). Given that HDAC5 and AurB colocalized during these cell cycle stages, and that HDAC phosphorylation was sensitive to AurB inhibition, we next determined whether AurB-dependent Ser278 phosphorylation levels are differentially regulated during cell cycle progression. Cell lines stably expressing HDAC5-EGFP were arrested in G1/S with hydroxyurea or G2/M with nocodazole, and the relative levels of pSer278 HDAC5 were examined (Fig. 5A). An increase in pSer278 HDAC5 levels was observed in G2/M when compared with asynchronous or G1/S cells. To assess the dependence of HDAC5 phosphorylation on AurB activity during G2/M, HDAC5-EGFP was immunoaffinity purified from G2/M cells treated with Hesperadin. Indeed, Ser278 phosphorylation in G2/M cells was sensitive to AurB inhibition (Fig. 5B). Furthermore, the physical association of AurB with HDAC5 was confirmed, as demonstrated for HDAC4 and HDAC9 (Fig. 3). Interestingly, this interaction was disrupted by Hesperadin treatment (Fig. 5B and supplementary Fig. S1).

To confirm the cell cycle-dependent regulation of NLS Ser278 phosphorylation, we next assessed this phosphorylation in endogenously expressed HDACs by immunofluorescence microscopy (Fig. 5C). WT HEK293 cells immunostained with anti-pSer278 showed a low level of reactivity within the nuclei of cells in interphase. However, in cells undergoing mitosis, a significantly greater fluorescent intensity was evident, demonstrating increased expression of endogenous HDAC phosphorylation (pSer278, pSer265 and pSer242 in HDAC5, HDAC4, and HDAC9, respectively) during mitosis. Additionally, phosphorylated HDACs appeared most prominently at the midbody, progressively decreasing toward the re-forming daughter nuclei and cell poles. Kapoor and colleagues have shown that AurB regulates phosphorylation of Ser278 in HDAC5, Ser265 in HDAC4, and Ser242 in HDAC9 in HEK293 or CEM T whole cell lysates.

FIG. 3. Conserved NLS Ser278 is a substrate for Aurora B phosphorylation in vitro. Phosphorylation assays were performed using recombinant human AurB and a synthetic NLS peptide (NLSpep). Serine phosphorylation status was monitored by single-stage MS and ETD MS/MS using an LTQ Orbitrap Velos instrument. A, Mass spectra of unmodified (left) and phosphorylated (right) NLSpep. Phosphorylated NLSpep was detected upon incubation with AurB and ATP (middle), but not in the absence of ATP (top) or presence of staurosporine (SSP) (bottom), *, contaminant ion. B, ETD tandem mass spectra confirmed that Ser278 was the sole phosphorylation site (bottom), as supported by c7 and z6 fragment ions (square boxes).

FIG. 4. Class Ila HDACs are in vivo substrates of Aurora B. A, An antibody generated against phosphorylated Ser278 specifically recognizes the phosphorylated form of wild type (WT) HDAC5-EGFP (HDAC5pS278) and not S278A or S278/279A HDAC5-EGFP phosphomutants, as shown by Western blotting of whole cell lysates from cell lines stably expressing EGFP alone, WT HDAC5-EGFP, or phosphomutants of HDAC5-EGFP. B, siRNA-mediated inhibition of AurB expression reduces Ser278 phosphorylation in HDAC5 in HEK293 whole cell lysates, Human AURKB (AurB) versus nontargeting (NT) esiRNA (final concentration 3.4 nM). *, band corresponding to combined signal of endogenous phosphorylated HDAC4, HDAC5, and HDAC9. C–E, AurB inhibition with Hesperadin abolishes phosphorylation of Ser278 in HDAC5, Ser265 in HDAC4, and Ser242 in HDAC9 in HEK293 or CEM T whole cell lysates.
Aurora B-dependent HDAC Phosphorylation is Complemented by Decreased Association of HDACs with the NCoR Complex During Mitosis—

To explore the functional significance of this AurB-dependent phosphorylation, phospho-specific Ser278 HDAC5-EGFP protein associations were assessed. Isolation of pSer278 HDAC5-EGFP in asynchronous cells via the anti-pSer278 antibody showed association with AurB, along with reduced 14-3-3 binding, as compared with HDAC5-EGFP isolated via anti-GFP (Fig. 6A). As Ser278 falls between 14-3-3 binding sites Ser259 and Ser498, it is conceivable that AurB and 14-3-3-dependent signaling are coordinately regulated. To explore this possibility, we examined AurB association with the Ser259/498Ala HDAC5-EGFP double mutant (S259/498A), which cannot bind 14-3-3. Compared with immunopurified WT HDAC5-EGFP, S259/498A showed greater AurB association and increased Ser278 phosphorylation (Fig. 6B). Interestingly, such a competition between AurB and 14-3-3 is not unprecedented; AurB and 14-3-3 coordinate phosphorylation of centralspindlin, which blocks 14-3-3 binding (45). It is possible that coordinated regulation of HDAC5 phosphorylation at 14-3-3-binding sites contributes to HDAC5 localization during mitosis. This coordinated regulation could be explained by two possible mechanisms: (1) 14-3-3 binding impedes AurB access to HDAC5 or (2) AurB-dependent phosphorylation of Ser278 impairs 14-3-3 binding. Interestingly, we observed no significant change in association of HDAC5 with 14-3-3 proteins after G2/M arrest compared with asynchronous cells, as measured by mass spectrometry-based spectral counting (Fig. 6C). Although this contrasts with our observations from isolated phosphorylated HDAC5 in asynchronous cells (Fig. 6A), the apparent disparity may be attributed to the fact that, in nondividing cells, pSer278 HDAC5 is present at lower abundance and localizes to the nucleus, where it would not be expected to interact with 14-3-3 proteins for export to the cytoplasm (Fig. 5C). Together, these results suggest that while phosphorylation of Ser278 alone is insufficient to disrupt 14-3-3 binding during mitosis, reduced 14-3-3 binding allows for increased phosphorylation of this site by AurB.
To further assess the functional significance of AurB-dependent HDAC5 phosphorylation in the context of cell cycle regulation, we investigated changes in HDAC5 interactions known to be involved in transcriptional regulation. We first employed label-free spectral counting analysis to assess changes in relative abundance of well-established class IIa HDAC interactions after G2/M arrest (NOC) compared with asynchronous (DMSO) cells. A minimum of 5 total spectra in each biological replicate (N ≥ 4) in at least one treatment condition (DMSO, NOC) was required for relative quantification. To correct for differences in immunosolated HDAC5 bait, prey protein spectral counts in the NOC replicates were normalized to respective HDAC5-EGFP spectral counts in the DMSO controls (supplemental Table S3). In agreement with Fig. 5B, these experiments (supplemental Fig. S2) further confirmed the association of HDAC5 with AurB in NOC-arrested cells, although AurB did not pass the spectra threshold for quantification, consistent with a transient association. Interestingly, we observed a decrease in HDAC5 associations with members of the NCoR complex: NCoR1, TBL1X, TBL1XR1, co-isolated from the mitotic cell populations relative to the asynchronous cell populations (Fig. 6C). This change in HDAC5 interaction with the NCoR complex was less prominent in a comparison of G1/S-arrested cells (by Hydroxyurea) with the asynchronous cell population (supplemental Fig. S3). To further confirm these changes in HDAC5 interactions during mitosis, we performed additional small-scale immunoaffinity purifications of HDAC5-EGFP from cells treated with DMSO or Nocodazole for Western blot analysis (n = 2 for each treatment). The decrease in HDAC5 association with NCOR1, TBL1X, and TBL1XR1 during mitosis was apparent, whereas the association with 14-3-3ε remained constant (Fig. 6D and supplemental Fig. S4). Interestingly, the inhibition of Aurora B with Hesperadin in the absence of any additional treatment led to a decrease in association of HDAC5 with the NCOR complex, as well as with Aurora B, although not affecting the interaction with 14-3-3ε (supplemental Fig. S5). This suggests that Hesperadin, known to block nuclear division and progression through cytokinesis, can disrupt the association of cellular factors critical for transcriptional regulation. Therefore, these different lines of evidence suggest a dynamic regulation of the transcriptional roles of HDAC5 during mitosis that possibly involves a cumulative effect of interactions and posttranslational modifications by phosphorylation. Tightly linked with its role in transcriptional repression is the deacetylation activity of HDAC5. When we compared HDAC5-EGFP deacetylation activity by in vitro assays, we observed a decrease in HDAC5 enzymatic activity after isolation during mitosis as compared with asynchronous cells (supplemental Fig. S6). Interaction of HDAC5 with the NCOR complex is required for its transcriptional repressive functions and deacetylation activity. Decreased association of HDAC5 with these nuclear interacting partners during mitosis could partly be due to the midzone localization of HDAC5 within a phosphorylation gradient. Thus, AurB-dependent phosphorylation of HDAC5 may indirectly serve to interrupt participation of HDAC5 with the NCOR complex during mitosis through a localization-dependent mechanism of protein sequestration, which may be accompanied by additional cell cycle-dependent changes in interactions within transcriptional complexes.

**CONCLUSIONS**

Here, we identify a new regulatory pathway for class IIa HDACs that fits within a model in which specific phosphorylations serve as regulatory switches to determine the functionally divergent fates of these enzymes (Fig. 7). NLS phosphorylation of class IIa HDACs by PKA (16) and Cdk5 (17) (e.g. Ser279 in HDAC5) is important for nuclear localization (15). In the nucleus, class IIa HDACs bind transcription factors and the NCOR complex, forming a multi-protein complex capable of repressing transcription of downstream gene targets. Alternatively, when phosphorylated by CaMK/PKD (1, 6, 12, 46–50), class IIa HDACs bind 14-3-3 chaperone proteins, which facilitates their nuclear export, thereby relieving HDAC-mediated transcriptional repression.

We demonstrate that class IIa histone deacetylases are previously undescribed substrates of Aurora B, an essential mitotic kinase. We identify the precise sites of Aurora B-de-
ependent phosphorylation within the nuclear localization signals of HDAC4, HDAC5, and HDAC9, and show that these sites are temporally and spatially regulated during cell cycle progression. This is the first time that HDAC phosphorylation is demonstrated to be dynamically regulated during cell cycle progression. We show that during late mitosis and cytokinesis, HDAC5 localizes to the spindle midzone and midbody. Furthermore, we show that endogenous phosphorylated HDACs are sequestered within an Aurora B-dependent phosphorylation gradient at the spindle midzone. A functional consequence of this localization is the separation of these deacetylases from re-forming daughter nuclei. Finally, we demonstrate that HDAC5 interactions with the nuclear corepressor complex, necessary for HDAC function in transcriptional repression, are significantly decreased during mitosis. Cumulatively, midzone sequestration of histone deacetylases and the accompanying changes in protein interactions can significantly contribute to cell cycle-dependent regulation of HDAC-mediated transcriptional repression.

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