Class IIa Histone Deacetylases Are Conserved Regulators of Circadian Function*

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Background: Class IIa HDACs are signal-dependent transcriptional corepressors that regulate cell differentiation programs and liver gluconeogenesis. Results: HDAC5 influences BMAL1 acetylation and interfering with normal expression levels of class IIa HDACs disrupts circadian rhythms. Conclusion: Class IIa HDACs regulate the robustness of cellular clocks and behavioral activity rhythms. Significance: Class IIa HDACs provide a conserved link between circadian clocks and metabolic signaling pathways.

Class IIa histone deacetylases (HDACs) regulate the activity of many transcription factors to influence liver gluconeogenesis and the development of specialized cells, including muscle, neurons, and lymphocytes. Here, we describe a conserved role for class IIa HDACs in sustaining robust circadian behavioral rhythms in Drosophila and cellular rhythms in mammalian cells. In mouse fibroblasts, overexpression of HDAC5 severely disrupts transcriptional rhythms of core clock genes. HDAC5 overexpression decreases BMAL1 acetylation on Lys-537 and pharmacological inhibition of class IIa HDACs increases BMAL1 acetylation. Furthermore, we observe cyclical nucleocytoplasmic shuttling of HDAC5 in mouse fibroblasts that is characteristically circadian. Mutation of the Drosophila homolog HDAC4 impairs locomotor activity rhythms of flies and decreases period mRNA levels. RNAi-mediated knockdown of HDAC4 in Drosophila clock cells also dampsens circadian function. Given that the localization of class IIa HDACs is signal-regulated and influenced by Ca²⁺ and CAMP signals, our findings offer a mechanism by which extracellular stimuli that generate these signals can feed into the molecular clock machinery.

Circadian rhythms govern diurnal variations in physiological functions, synchronizing behavior to the 24-h cyclical changes in our environment. At the molecular level, circadian clocks involve periodic changes in gene expression achieved by transcription-translation feedback loops whereby the protein products of transcribed genes auto-regulate their own transcription. In mammals, the core transcriptional circuit is comprised of the transcription factors CLOCK and BMAL1 that heterodimerize and activate transcription of Period (Per) and Cryptochromes (Cry) genes. PER/CRY proteins then repress their own transcription by inhibiting the activity of CLOCK-BMAL1 until they are degraded to allow a new cycle of transcription to begin (1). In addition, an interlocking feedback loop regulates rhythmic expression of Bmal1 through opposing actions of the ROR and REV-ERB families of orphan nuclear receptors that activate and repress Bmal1 transcription, respectively, and whose expression is controlled by the core loop (1–3). This mechanism is conserved in the Drosophila core loop, where heterodimers of CLOCK and CYCLE induce transcription of period and timeless and the interlocking loop generates rhythmic changes in clock expression (4). These transcriptional oscillations are regulated by many post-translational events, including reversible protein acetylation that controls circadian gene expression by impinging on both transcription factor activity and chromatin structure via modification of histone proteins. Rhythmic histone acetylation has been observed at promoters of core clock genes (5) and at promoters of clock-controlled output genes (6). Additionally, many core components of the molecular clock, including BMAL1 and PER2, show daily oscillations in their acetylation status (7, 8). These rhythms in acetylation are generated by cellular histone acetyltransferases and histone deacetylases (HDACs).³ CLOCK-BMAL1 heterodimers recruit the transcriptional coactivators p300 and CREB-binding protein, which possess histone acetyltransferase activity (5, 9). Moreover, CLOCK itself has been reported to possess intrinsic histone acetyltransferase activity (10). In mammals, SIRT1 has been implicated in opposing the activity of histone acetyltransferases to regulate rhythmic acetylation of BMAL1 (7), PER2 (8), and histone H3 (8) in response to cellular energy levels. Class IIa histone deacetylases are related HDACs whose subcellular localization is regulated

³ The abbreviations used are: HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; SUMO, small ubiquitin-like modifier; NAM, nicotinamide; LD, light/dark cycle.
Class IIa HDACs Regulate Circadian Clocks

by extracellular stimuli via the second messengers Ca²⁺ and cAMP (11). In fact, many SIRT1 substrates also interact with class IIa HDACs. For example, in response to nutrients SIRT1 deacetylates FOXO (12) but in response to hormone signaling, FOXO deacetylation is mediated by interactions with class IIa enzymes (13, 14). Class IIa HDACs and SIRT1 both interact with MEF2 transcription factors (15) and HIC-1 (hypermethylated in cancer 1; 16) to coordinate their deacetylation and SUMOylation.

Mammalian class IIa HDACs lack intrinsic enzymatic activity and instead mediate deacetylation of proteins via recruitment of corepressor complexes containing HDAC3, a class I HDAC, and the nuclear receptor corepressors NCoR and SMRT (silencing mediator of retinoic and thyroid hormone receptors) (17). For example, HDAC4 recruits the nuclear corepressor NCoR and HDAC3 to deacetylate FOXO transcription factors (14). The recruitment of SMRT/NCoR-HDAC3 complexes by class IIa HDACs could also affect histones and influence chromatin (18). Given that class IIa HDACs have the potential to influence rhythms of gene expression through their effects on both histones and non-histone proteins, we investigated their role in circadian function.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—Expression vectors for wild-type HDAC5-FLAG, wild-type HDAC5GFP (HDAC5WT), and GFP-fused HDAC5 mutant (HDAC5MUT) have been described previously (19). The luciferase reporter plasmids contain either the mouse Bmal1 promoter (Bmal1::luc; 20) or the mouse Per2 promoter (Per2::luc; 20) upstream of firefly luciferase gene. Plasmids encoding FLAG-tagged mouse CLOCK (FLAG-CLOCK) and Myc-tagged mouse BMAL1 (BMAL1-Myc) were kindly provided by Dr. Nicolas Cermakian (McGill University) and have been described (21). Primary antibodies against phospho-HDAC4/5 Ser-246/259, acetyl histone H3(K9), histone H3, and the FLAG epitope were obtained from Cell Signaling Technology. Anti-acetyl BMAL1(Lys-537) and anti-CLOCK were from Millipore, and anti-HDAC5 made in goat was purchased from Santa Cruz Biotechnology.

Cell Culture and Bioluminescence Measurements—NIH3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Glutamax, and penicillin/streptomycin (all from Invitrogen). Cells were transfected 24 h after plating using Gene Juice (Novagen, San Diego, CA) according to the manufacturer’s instructions. Plasmids were transfected with the indicated expression constructs in a ratio 7:2:1 expression vector:circadian (firefly) luciferase reporter:pRL-CMV (Renilla luciferase, Promega). Renilla luciferase activity was used as an internal control to correct for transfection efficiency. Cells were synchronized by replacing the medium with air medium and sealing the dishes prior to bioluminescence recordings, which were performed using custom-made photomultiplier assemblies housed in a 37 °C incubator as described previously (22).

Drosophila Stocks and Behavioral Assays—All fly stocks were maintained on standard yeast-sugar-agar food. The HDAC5K09091 hypomorph mutant (13) was obtained from the Bloomington Stock Center (Indiana University). UAS-HDAC4RNAi (VDRC 20522) strain was obtained from the Vienna Drosophila RNASi Center (Vienna, Austria). The y wtim-Gal4 driver line (23) was obtained from Professor Ralf Stanewsky (Queen Mary, University of London). A DAM2 Drosophila activity monitor system (Trikinetics, Inc., Waltham, MA) was used to record locomotor activity in 2-min bins. 1-to-4-day-old adult males were collected and loaded into activity tubes containing 5% sucrose in 1% agar food at one end. Flies were entrained to 12- to 12-h light/12-h dark cycles (LD) at 25°C for 3 days and then monitored in constant darkness for 7 to 10 days. Activity records were analyzed using ActogramJ (24), and circadian rhythmicity was assessed by Lomb-Scargle periodogram analysis of the constant darkness data.

Drosophila RNA Analysis—2- to 4-day-old males were entrained for 3 days in LD conditions and then frozen at the indicated zeitgeber times. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase. 1 µg of RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen), and the resulting cDNA was amplified with gene-specific primers for semi-quantitative PCR analysis. Primer sequences for Drosophila genes were as follows: period, 5′-CGTGCTGTGTCTG-GTCCCTC-3′ (forward) and 5′-ACGGACAGCAATTGGGAA-TAG-3′ (reverse); 18S rRNA, 5′-AACATGAACCTTATGGG-ACATGTG-3′ (forward) and 5′-TCGGTACACATACATC-GATCTGC-3′ (reverse); and HDAC4, 5′-ACAACCGCTCCAG-TAACCCT-3′ (forward) and 5′-CCAGTGTCGGGAATCT- GACT-3′ (reverse).

Western Blotting—To assess the effect of HDAC inhibitors on BMAL1 acetylation, NIH3T3 cells transfected with BMAL1-Myc using FuGene 6 (Roche Applied Science) were treated with indicated HDAC inhibitors 24 h after transfection. After 18 h, cells were lysed in hot SDS sample buffer. To assess the effect of HDAC5 expression on BMAL1 acetylation protein extracts were prepared from cells 40 h after transfection. Proteins were resolved by SDS-PAGE using pre-cast Bis-Tris gels (Invitrogen) and immunoblotted using standard protocols. Band intensities were quantified with ImageJ software. Statistical significance was determined with a two-tailed paired Student’s t test using the SPSS Statistical suite (IBM).

HDAC5 Localization—HDAC5 subcellular localization was assessed by live cell imaging or immunofluorescence in near confluent cultures. For live cell imaging, HDAC5GFP-transfected NIH3T3 cells were imaged 24 h after transfection. Cells were imaged at 37 °C on a Zeiss laser scanning confocal system (Zeiss LSM 780 multiphoton) in medium (DMEM containing 10% FBS and penicillin/streptomycin) that was replaced immediately before the start of the experiment. Images of GFP-transfected cells were captured every 2 h for 2 consecutive days. For immunofluorescence, NIH3T3 cells were fixed at indicated times after synchronization of cultures with 10 µM forskolin. Cells were fixed in 3% paraformaldehyde/4% sucrose in PBS for 20 min. Cells were subsequently washed in PBS followed by permeabilization in 0.5% Nonidet P-40 in PBS. Cells were incubated with phospho-HDAC4/5 antibody (diluted 1:100) overnight at 4°C. Alexa Fluor-conjugated secondary antibodies were used at 1:500 and nuclei were visualized with a Hoechst stain. Images were captured on a Zeiss laser scanning confocal system, and immunofluorescence intensities were assessed using ImageJ software.
Class IIa HDACs Regulate Normal Circadian Function—

Class IIa HDACs shuttle between the nucleus and the cytoplasm in response to extracellular signals. Their cytoplasmic localization is mediated by phosphorylation of two conserved serine residues in their N termini (serines 259 and 498 in HDAC5) that promotes their interaction with 14-3-3 proteins. To examine whether class IIa HDACs play a role in modulating circadian rhythms, we examined the effect of overexpressing HDAC5 (HDAC5WT) that promotes their interaction with 14-3-3 proteins. Phospho-Ser-259 immunoreactivity co-localizes with HDAC5 expression was detected with a FLAG antibody, and HDAC5 Ser-259 phosphorylation was assessed using a phospho-HDAC4/5/7 antibody. Phospho-Ser-259 immunoreactivity co-localizes with HDAC5 and is detected in the nucleus of untreated FLAG-HDAC5WT transfected cells. Phorbol 12-myristate 13-acetate stimulation induces HDAC5 nuclear export, and FLAG and phospho-HDAC5 immunoreactivity can be seen in the cytoplasm of FLAG-HDAC5WT transfected cells. FLAG-HDAC5MUT does not translocate to the cytoplasm in response to phorbol 12-myristate 13-acetate due to mutation of serines 259 and 498 and is not detected by the phospho-HDAC4/5/7 antibody. Scale bar is 10 μm.

RESULTS

Class IIa HDACs Regulate Normal Circadian Function—

Class IIa HDACs shuttle between the nucleus and the cytoplasm in response to extracellular signals. Their cytoplasmic localization is mediated by phosphorylation of two conserved serine residues in their N termini (serines 259 and 498 in HDAC5) that promotes their interaction with 14-3-3 proteins. To examine whether class IIa HDACs play a role in modulating circadian rhythms, we examined the effect of overexpressing both wild-type HDAC5 (HDAC5WT) and a phosphorylation-defective mutant (HDAC5MUT) (19) that is rendered constitutively nuclear due to mutation of the two conserved serines to alanines. Fig. 1 shows that the HDAC5WT is nuclear in untreated NIH3T3 cells and translocates to the cytoplasm when cells are stimulated with phorbol 12-myristate 13-acetate, whereas HDAC5MUT is constitutively nuclear. We examined the transcriptional rhythms of Bmal1 and Per2 in NIH3T3 mouse fibroblasts by co-expressing HDAC5 with luciferase reporter plasmids where the luciferase gene is driven either by the Bmal1 promoter (Bmal1::luc) or the Per2 promoter (Per2::luc). Real-time bioluminescence measurements show that HDAC5 overexpression severely compromised Bmal1 transcriptional rhythms (Fig. 2A). Compared with control cells that displayed clear rhythmic Bmal1 luciferase luminescence oscillations for four cycles, circadian rhythms were lost after the first cycle in cells expressing HDAC5WT and completely abolished in cells expressing the constitutively nuclear HDAC5MUT (Fig. 2A). Both HDAC5WT and HDAC5MUT dampened the amplitude of Per2 transcriptional oscillations (Fig. 2B and C) without affecting the period (unpaired t test compared with control, p > 0.5, n = 6). Expression of wild-type HDAC5 caused a significant reduction in the amplitude of Per2 luciferase bioluminescence in the first cycle, whereas there was little effect of HDAC5MUT expression. However, both the wild-type and mutant HDAC5 proteins caused a similar reduction in Per2 luciferase amplitude and magnitude in successive cycles with an increased rate of dampening (Fig. 2C).

We next investigated whether class IIa HDACs play a conserved role in sustaining normal circadian rhythms. Because many components of the core transcriptional-translational feedback loop are conserved in Drosophila, we examined loco-
motor activity rhythms in Drosophila lines with reduced expression of the fly homolog HDAC4. We used a HDAC4 hypomorph mutant line (HDAC4\(^{KG09091}\)) containing a P-element insertion in the 5′/H11032-UTR, which leads to reduced HDAC4 mRNA levels (Fig. 3A). We assessed locomotor activity in HDAC4\(^{KG09091}\) flies that were out-crossed with the wild-type strain, Canton-S and compared these with control flies generated by crossing the ry506 strain with Canton-S. The mutant flies showed normal locomotor activity profiles and circadian rhythms in 12-h LD cycles (Fig. 3B). However, in free-running conditions of constant darkness, 41% of HDAC4 mutant flies lacked any rhythms. Fig. 4A shows representative actograms of arrhythmic HDAC4 mutant flies and control flies. Lomb-Scargle periodogram analysis revealed that the remaining HDAC4 mutant flies that showed rhythmic behavior exhibited weak rhythms with a 60% decrease (Fig. 4B) in the power (a measure of the strength) of the rhythm. Although HDAC4 mutants displayed less robust rhythms, there was no significant difference in the period (Fig. 4C). To investigate whether HDAC4 knockdown in Drosophila affects the molecular clock that underlies behavioral rhythms, we tested the effects of HDAC4 gene disruption on period mRNA levels. HDAC4 mutant flies had reduced period mRNA expression in LD cycles at ZT16 but had comparable period expression at ZT8 (Fig. 4D).

Because HDAC4 is involved in many functions, including differentiation of Drosophila muscle, we used the Gal4/UAS system to direct RNAi-mediated knockdown of HDAC4.
expression. We crossed transgenic flies that express RNAi directed against HDAC4 driven by a UAS element (UAS- HDAC4RNAi) with those that express Gal4 driven by the timeless gene promoter (tim-Gal4) to specifically knockdown HDAC4 RNA levels in all clock cells. Table 1 shows that Drosophila with reduced expression of HDAC4 in clock cells have either disrupted rhythms (31% arrhythmic flies) or weaker circadian activity rhythms, but the period remains unaffected. This is consistent with the behavioral phenotype of HDAC4KG09091 mutant flies. Taken together, our experiments in mammalian cells and Drosophila lines indicate that interfering with normal levels of class Ila HDAC expression disrupts circadian function at both the cellular and behavioral level.

**HDAC5 Influences BMAL1 Acetylation**—We next investigated whether class Ila HDACs can, similar to SIRT1, influence acetylation of BMAL1. We treated BMAL1-Myc transfected NIH3T3 cells with various HDAC inhibitors and assessed BMAL1 acetylation using an antibody specific for lysine 537-acetylated BMAL1. Trichostatin A, the class I and class II HDAC inhibitor increased BMAL1 acetylation levels 1.85-fold (Fig. 5, A and B). Trichostatin A had a dramatic effect on histone H3 Lys-9 acetylation, increasing it by 6.9-fold, a consequence of its inhibitory action on class I HDACs. The class II-specific HDAC inhibitor MC1568 (25) also increased BMAL1 acetylation, and the effect was similar to the class III HDAC inhibitor nicotinamide (NAM). MC1568 and NAM had an additive effect when applied together. MC1568 increased H3 Lys-9 acetylation to a lesser degree than trichostatin A, whereas NAM did not influence histone H3 acetylation. Although pharmacological inhibition of class II HDACs increased BMAL1 acetylation, we found that co-expression of either wild-type HDAC5 or the constitutively nuclear HDAC5 mutant with BMAL1-Myc decreased BMAL1 acetylation (Fig. 5, C and D). The wild-type and mutant HDAC5 were equally effective at deacetylating BMAL1.

The subcellular localization of BMAL1 changes during circadian cycles, and the nucleocytoplasmic shuttling of BMAL1 is abolished in fibroblasts with disrupted circadian clocks (26). We therefore examined the effect of HDAC5 co-expression on the subcellular localization of ectopically expressed BMAL1. In agreement with previous work (27), we found that BMAL1 was nuclear when expressed alone (data not shown). In contrast, the cellular localization of BMAL1 in HDAC5-expressing cells was heterogeneous, and BMAL1-Myc and HDAC5 could be found co-localized either in the cytoplasm (Fig. 5E, top panel), or in the nucleus (Fig. 5E, bottom panel), or in both the nucleus and cytoplasm (Fig. 5E, middle panel). Interestingly, when BMAL1 and HDAC5 were exclusively nuclear or exclusively cytoplasmic BMAL1 acetylation was abolished (Fig. 5E, top and bottom panel). This suggests that classically located wild-type HDAC5 prevents BMAL1 acetylation by sequestering it in the cytoplasm and nuclear HDAC5 (wild-type or mutant) likely recruits deacetylating enzymes such as HDAC3 to BMAL1. This would explain why wild-type and mutant HDAC5 influence BMAL1 acetylation to a similar extent. These data suggest that class Ila HDACs have a functional role in regulating BMAL1 acetylation.

**HDACs Shows Circadian Changes in Subcellular Localization**—BMAL1 acetylation has been shown to display a circadian profile (7). Our experiments above show that class Ila HDACs can influence BMAL1 acetylation but do not explain how HDAC5 might confer circadian periodicity to BMAL1 acetylation. Because class Ila HDACs shuttle in and out of the nucleus in a signal-dependent manner, we investigated whether HDAC5 shows oscillatory changes in its nuclear and cytoplasmic localization. To do this, we examined the localization of an HDAC5-GFP fusion protein in live cells by time-lapse fluorescence microscopy. Images of transfected NIH3T3 cells were captured every 2 h, and the ratio of GFP fluorescence in the nucleus and cytoplasm was assessed. Fig. 6 shows an example of a typical cell in which HDAC5 shuttles in and out of the nucleus with a circadian profile. The nuclear/cytoplasmic ratio in this cell (cell 1, Fig. 6B) shows ~24 h of cyclical changes. The nuclear/cytoplasmic ratio for two other cells is shown in the graph (Fig. 6B), with only partial synchronization being observed following media change, as was reported previously for uncoupled fibroblast bioluminescence rhythms (28). Because the nuclear export of HDAC5 has been reported to correlate with an increase in phosphorylation at series 259 and 498, we used a phospho-Ser-259 specific antibody to examine whether the phosphorylation status of HDAC5 shows rhythmic changes and correlates with its cytoplasmic localization. Fig. 6C shows phospho-HDAC5 Ser-259 immunoreactivity in forskolin-synchronized NIH3T3 cells fixed every 4 h after synchronization. Surprisingly, phospho-Ser-259 immunoreactivity is detectable in the nucleus during this time course (Fig. 6C). Strikingly, phosphorylated HDAC5 shuttles in and out of the nucleus with a 24-h period: it is nuclear at time 0, but exclusively cytoplasmic at 8 h and again at 36 h after synchronization (Fig. 6C). To verify that the observed nuclear immunoreactivity detected with the phospho-Ser-259 is due to HDAC5, we validated that the antibody only recognizes expressed HDAC5WT, and no signal is detectable in cells transfected with the non-phosphorylatable mutant HDAC5MUT (Fig. 1). The phospho-Ser-259 antibody detected both nuclear and cytoplasmic HDAC5WT in NIH3T3 cells (Fig. 1). The changes in localization of HDAC5-GFP in live cells and the temporal profile of phospho-Ser-259 HDAC5 immunoreactivity together indicate a cyclical variation in HDAC5 subcellular localization that does not depend on changes in Ser-259 phosphorylation. The time of day-dependent presence of HDAC5 in the nucleus could account for circadian changes in the acetylation status of BMAL1.
Class IIa HDACs Regulate Circadian Clocks

DISCUSSION

Rhythmic changes in the acetylation status of core clock transcription factors and of histones at gene promoters play an important role in imparting circadian periodicity to physiological processes such as metabolism. In this context, the contribution of several histone acetyltransferases and HDACs has been studied. Among the HDAC family, the class I enzyme HDAC3 has been shown to control circadian expression of liver genes involved in lipid biosynthesis (6), and the class III NAD-activated enzyme HDAC3 has been shown to control circadian expression of liver genes involved in lipid biosynthesis (6). In this regard, it is noteworthy that mouse BMAL1 is SUMOylated on Lys-259, a site conserved in vertebrates but absent in invertebrates. HDAC5 expression appears to have a more severe circadian phenotype than SIRT1 expression. This is backed by observations from mouse fibroblasts, HDAC4 mutants. Notably, we find that Drosophila lines with reduced expression of the SIRT1 homolog Sir2 show no circadian defects in behavioral rhythms (data not shown). The dampened Per2 transcriptional rhythms that persist with unaltered periodicity in the face of severely disrupted Bmal1 transcriptional rhythms in HDAC5-transfected fibroblasts suggest that the ROR/REV-ERB-dependent interlocking feedback loop is not required for determining the period of circadian oscillations and is in agreement with previous work (3). Importantly, this accessory loop modulates the amplitude and magnitude of transcriptional rhythms in the core circuit, and this likely accounts for the less robust behavioral rhythms seen in Drosophila HDAC4 mutants.

At the molecular level, we find that HDAC4/5 inhibition led to increased acetylation of BMAL1 on Lys-537, and HDAC5 overexpression reduced BMAL1 acetylation. This effect of HDAC5 overexpression on BMAL1 acetylation would be expected to result in a loss of rhythmic expression of CLOCK/BMAL1-controlled target genes, including members of the REV-ERB and ROR family of proteins and could account for the deregulated Bmal1 transcriptional rhythms seen in HDAC5-overexpressed cells. The acetylated residue in mouse BMAL1 (Lys-537) is highly conserved in vertebrates but is absent in Drosophila homolog CYCLE, raising questions about how class IIa HDACs affect the molecular oscillator in Drosophila. It is possible that the observed acetylation/deacetylation of this residue in our HDAC5 inhibition/overexpression experiments reflects a loss of cellular rhythms mediated by molecular events unrelated to BMAL1 acetylation. Class IIa HDACs can act as E3 SUMO ligases and promote SUMOylation of several proteins. In this regard, it is noteworthy that mouse BMAL1 is SUMOylated on Lys-259, a

FIGURE 5. HDACs influences BMAL1 acetylation and interacts with CLOCK-BMAL1. A, representative Western blot showing the effect of HDAC inhibitors on BMAL1 acetylation at Lys-537 and H3 acetylation at Lys-9. 24 h after transfection, cells were left untreated (Con) or incubated with indicated HDAC inhibitors: 2 μM trichostatin A (TSA) or 10 μM MC1568 (MC), or 10 μM NAM, or 10 μM MC1568 and 10 μM NAM together (N/M). After 18 h, cells were processed for immunoblotting using anti-acetyl Lys-537 BMAL1 antibody. Immunoblots were stripped and sequentially probed with an antibody to Myc, followed by an anti-acetyl H3 and finally an anti-histone H3 antibody. B, quantitative analysis of BMAL1 acetylation and H3K9 acetylation from immunoblots in A is shown in the graph. The immunoreactivity was measured using imageJ software, and acetyl-BMAL1 immunoreactivity was normalized to the corresponding BMAL1-Myc level, and H3K9 immunoreactivity was normalized to that of H3. Values are shown as mean ± S.E., and data are from three independent experiments. Double asterisks indicate significant increase in BMAL1 acetylation when compared with the control (Con); p < 0.01 (Student’s t test). A single asterisk indicates significant increase in BMAL1 acetylation when compared with the control (Con), p < 0.05 (Student’s t test). C, representative Western blot showing the effect of HDAC5WT and HDAC5mut expression on BMAL1 Lys-537 acetylation. D, graph showing quantitative analysis of immunoblots in C. Data are from four independent transfection experiments and is shown as mean ± S.E. A single asterisk indicates significant reduction in BMAL1 acetylation when compared with the control p < 0.05 (Student’s t test). E, representative examples of cells showing localization of HDAC5-FLAG and BMAL1-Myc when co-expressed. BMAL1 acetylation was assessed in cotransfected cells, and the nucleus is identified by Hoechst staining. Scale bar is 10 μm.
Class IIa HDACs Regulate Circadian Clocks

Residue that is conserved in *Drosophila* Cycle (29). An interplay between HDAC4/5-mediated SUMOylation and SIRT1-mediated deacetylation has been described for MEF2 (15) and HIC1 (16). Interestingly, MEF2 proteins have been implicated in controlling circadian behavior in *Drosophila* (30).

It is therefore likely that HDAC5 co-exists with SIRT1 in a regulatory complex with CLOCK-BMAL1 acting in concert to influence the core oscillator. Further studies will be required to illuminate the molecular basis for the conserved action of class IIa HDACs in mammalian and *Drosophila* clocks.

Signal-regulated nucleocytoplasmic shuttling is a distinct trait of class IIa HDACs that sets them apart from other HDAC family members. Here, we show that HDAC5 shows cyclical changes in its localization oscillating between the nucleus and cytoplasm with circadian timing. Of note, this nucleocytoplasmic shuttling appears to be independent of HDAC5 phosphorylation at the conserved serine 259. The antibody used here that detects phospho-HDAC5 Ser-259 would also recognize Ser-246 phosphorylated HDAC4, and it is possible that HDAC4 localization also changes with circadian timing. Depending on the cell type and the signal, phosphorylation of class IIa HDACs can be mediated by Ca<sup>2+</sup>/calmodulin-dependent kinases (CaMKII and CaMKIV), protein kinase D, salt-inducible kinases, and AMPK kinases and in most instances phosphorylation of HDAC5 on serines 259 and 498 leads to its nuclear exclusion (11). However, phospho-Ser-259-independent nuclear accumulation of HDAC5 has been reported in Cos7 cells where elevated levels of cellular cAMP inhibit phorbol 12-myristate 13-acetate-induced nuclear export of HDAC5 even when Ser-259 is phosphorylated (31). This raises the possibility that rhythmic changes in cellular cAMP levels, an integral feature of the core circadian oscillator (32), drive intrinsic oscillations in HDAC5 nucleocytoplasmic shuttling. In addition to regulating subcellular localization, phosphorylation of HDAC5 could also influence its interactions with other proteins such as class I HDACs. Sequential phosphorylations on distinct sites could regulate dissociation of HDAC5 from class I HDACs followed by 14-3-3 binding and subsequent nuclear export. This would explain why BMAL1 can be acetylated or deacetylated in the presence of wild-type nuclear HDAC5 (Fig. 5E, compare middle and bottom rows).

Nucleocytoplasmic shuttling of class IIa HDACs also occurs acutely in response to hormones and is involved in regulating gluconeogenesis in mammals (14) and in *Drosophila* (13). In the liver, the fasting hormone glucagon causes class IIa HDACs to accumulate in the nucleus where they deacetylate and activate FOXO transcription factors to induce expression of gluconeogenic genes. Insulin, on the other hand, promotes nuclear exclusion of HDAC4/5 by inducing their phosphorylation (13, 14). The self-sustained cell autonomous oscillations in HDAC5 localization that we have observed in NIH3T3 fibroblasts raise the possibility that the liver may be able to control glucose production with circadian periodicity in the absence of hormone signals or feeding-fasting cues. Our work thus adds class IIa HDACs to a growing list of proteins that are involved in mediating both evoked cellular changes in response to metabolic cues as well as sustaining cellular circadian cycles, allowing metabolic processes to feed back to the clock.

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