The impact of histone deacetylases (HDACs) in the control of gonadotropin releasing hormone (GnRH) neuronal development is unknown. We identified an increase in many HDACs in GT1-7 (differentiated) compared with NLT (undifferentiated) GnRH neuronal cell lines. Increased HDAC9 mRNA and protein and specific deacetylase activity in GT1-7 cells suggested a functional role. Introduction of HDAC9 in NLT cells protected from serum withdrawal induced apoptosis and impaired basal neuronal cell movement. Conversely, silencing of endogenous HDAC9 in GT1-7 cells increased apoptosis and cell movement. Comparison of WT and mutant HDAC9 constructs demonstrated that the HDAC9 pro-survival effects required combined cytoplasmic and nuclear localization, whereas the effects on cell movement required a cytoplasmic site of action. Co-immunoprecipitation demonstrated a novel interaction of HDAC9 selectively with the Class IIb HDAC6. HDAC6 was also up-regulated at the mRNA and protein levels, and HDAC6 catalytic activity was maintained in differentiated GnRH neuronal cells.

During embryogenesis gonadotropin releasing hormone (GnRH) neurons migrate from the olfactory placode to the forebrain. A myriad of factors can influence the process of GnRH neuronal development, and any failure in survival or migration of these neurons can lead to incomplete or absent sexual maturation (1–3).

A growing number of studies suggest that neuronal development may be controlled epigenetically (4, 5). Epigenetic changes are governed by a variety of enzymatic modifications including DNA methylation and histone modification. Histone deacetylases (HDACs) are a family of proteins initially described for their role in deacetylating histone tails acting as co-repressors to gene transcription. Whereas classes I HDACs are ubiquitous nuclear modulators of gene expression, the other HDAC classes (II-IV) have diverse, compartment-specific roles in normal physiology and pathophysiologic states (6, 7).

Class II HDACs are subdivided into IIA (HDAC 4, 5, 7, and 9) and IIB (HDAC6 and -10) (8, 9). The N-terminal regulatory domain mediates protein-protein interactions in the nucleus with tissue-specific partners, and the C-terminal domain contains the deacetylase activity (10). Class IIA HDACs display tissue-specific expression and contribute to differentiation and development (11). HDAC9 is highly expressed in striated muscle, where it acts as a negative regulator of differentiation and growth (12–14), and in brain where it is linked to neuronal morphogenesis (15). Based upon its sequence, HDAC9 is predicted to have weak HDAC activity, often requiring a partner to mediate its effects as a transcriptional modulator (16). The ability of Class IIA HDACs to shuttle from the nucleus to the cytoplasm also suggests they may have additional roles beyond their function as transcriptional co-repressors. Class IIB HDACs include a unique member, HDAC6, that has two-deacetylase domains. HDAC6 is a predominant cytoplasmically localized protein that has been shown to control rates of cell death via trafficking misfolded ubiquitinated proteins as well as foster
Novel Interaction of HDAC6 and HDAC9 in GnRH Neurons

The potential developmental role for modulating reproductive function.

Experimental Procedures

Plasmids—FLAG-pcDNA3-HDAC6 was a gift from Eric Verdin (Addgene plasmid #13823). Enhanced GFP (EGFP)-tagged mouse HDAC9 overexpressing plasmid (EGFP-HDAC9) and its translocating deficient mutant (EGFP-S218A/S448A-Hdac9) were provided by Noriyuki Sugo (Osaka University, Japan). pcMV 3Tag6 vectors inserted with FLAG-tagged full-length HDAC9 cDNA construct (amino acids 1–1069), truncated FLAG-tagged N-terminal HDAC9 construct (amino acids 1–636), and FLAG-tagged C-terminal HDAC9 construct (amino acids 637–1069) were a generous gift provided by Tapan Verdin (Addgene plasmid #13823). Sequences for all plasmids were confirmed using DNA sequencing (University of Colorado Denver, Cancer Center Sequencing Core Facility).

Antibodies and Reagents—HDAC6 (CO226) antibody was purchased from Millipore (Billerica, MA) and HDAC9 antibody (AP1109a) was obtained from Abgent (San Diego, CA). HDAC9 antibody (ab18970) antibody was purchased from Abcam (Cambridge, MA). For immunofluorescence experiments, rabbit anti-HDAC9 antibody (AP1109a) was obtained from Abgent (San Diego, CA). Donkey-anti-rabbit-FITC was purchased from Jackson ImmunoResearch (103161). GAPDH and β-tubulin antibodies were purchased from Millipore (Billerica, MA) and Abcam, respectively. Anti-α-tubulin (2144) and anti-caspase 3 antibody (9662) were purchased from Cell Signaling Technology (Beverly, MA). Acetylated α-tubulin (6-11B-1), BCL2 (7382), protein A/G Plus-agarose immunoprecipitation reagent, normal mouse, and rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Bio-Rad. Anti-FLAG mouse antibody for immunoblotting (F3165) and immunofluorescence (F4049) and anti-HA antibody (H6908) were purchased from Sigma. BAX antibody (BMS-163) was obtained from Bender MedSystems (Austria). Lamin was purchased from Cell Signaling. Tubastatin A was provided by T. McKinsey.

Cell Culture and Transfections—NLT (18) and GT1-7 GnRH cell lines (19) were cultured as previously described (20). Overexpression of vector and HDAC9 constructs in NLT cells were performed using Lipofectamine Plus reagent (Invitrogen) as per the manufacturer’s instructions. For silencing experiments, scrambled siRNAs and stealth siRNA pool for HDAC9 (Invitrogen) and HDAC6 siRNA (Ambion, NY) targeting HDAC9 and HDAC6, respectively, were transfected in GT1-7 cells using Lipofectamine 2000 (Invitrogen, CA). Cells were evaluated in functional assays 24–48 h post-transfection.

Adenoviral Transductions—Adenoviruses containing FLAG-tagged WT-HDAC6 and HDAC6 catalytic domain mutants (H216A/DD1 mutant, H611A/DD2 mutant, and H216A/H611A/DD1+DD2 mutant) were used. HDAC6 catalytic mutants H216A/DD1 (H611A/DD2) have single mutations at His-216 to Ala and His-611 to Ala. Double catalytic HDAC6 mutants (DD1+DD2) contain two mutations, His-216 to Ala and His-611 to Ala. For immunoprecipitation (IP) experiments, NLT cells were first transfected with FLAG-tagged vector and full-length HDAC9 constructs for 17 h followed by adeno viral transductions with FLAG tagged WT-HDAC6 and HDAC6 catalytic domain mutants (DD1, DD2, and DD1+DD2) at a multiplicity of infection of 25 pfu/cell. Cells were harvested 24 h post transduction and used for IP experiments.

Microarray—Total RNA was isolated from three separate aliquots of NLT and GT1-7 cells, and DNA microarray was performed. Bioinformatics analysis of the data were performed as previously described (21). Data were analyzed using GeneSpring software (Agilent Technologies, Santa Clara, CA) and Ingenuity Pathway Analysis (Redwood City, CA).

RT-PCR—RNA from hypothalamic, pituitary, and ovarian tissues of female adult mice were extracted using TRIzol reagent as previously described earlier (22). Total RNA from NLT and GT1-7 cells was extracted using RNeasy kit (Qiagen, Valencia, CA). RT was performed using iScript (Bio-Rad), and qPCR was performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster city, CA) in a real time PCR system as described earlier (23). The primer sequences used to amplify Hdac9 were 5′-CCATTGCCCACGTGAACC-3′ and 5′-TTCAAGTCATCGACTGATG-3′, and Hdac6 were 5′-TGCCGGACTAGAAGGACCC-3′ and 5′-GAAGGGGTACGTGCGATTG-3′, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were 5′-CGACCGCTCCATTGACCTCA-3′ and 5′-CCACGATACATCTAGACCC-3′. The cycle threshold values (Ct) obtained for Hdac9 and Hdac6 genes were normalized against Gapdh to calculate ΔΔCt values from triplicate experiments.

Immunoblot and IP—Immunoblotting of neuronal cell lysates were performed as previously described (23). Densitometry anal-
ysis using GAPDH as the internal loading control from three separate experiments was performed with the Bio-Rad Fluor-S multi-imager and NIH Image J software. The IP experiment was performed as previously described (21). For Tubastatin A effects, GT1-7 cells were treated with Tubastatin A (1 μM) for 24 h followed by harvesting and immunoprecipitation with HDAC6.

**HDAC Activity Assay**—Neuronal cell lysates were treated with acetylated HDAC substrates (I-1885 for Class I HDACs, I-1875 for Class IIb HDACs), and HDAC activity was determined as described (24). For HDAC activity assay, IP complexes were washed five times with HDAC assay buffer. The raw fluorescence signal was corrected for background, and data from three separate experiments were analyzed for significance.

**Migration Assay**—24 h after transfection, transfected NLT and GT1-7 cells were serum-starved for 48 h and 16 h, respectively. Cells were harvested and used for immunoblotting with cleaved caspase 3. For Hoechst staining transfected NLT and GT1-7 neuronal cells were plated on coverslips in serum-free medium for 16 h, then fixed and stained with Hoechst stain (33258) for 30 min (13). Apoptotic cells (with condensed or fragmented chromatin) from 8 randomly chosen fields were counted in 1000 cells from duplicate coverslips in 3 separate experiments using a fluorescent microscope (Axiovert 200 Zeiss microscope, Carl Zeiss, Oberkochen, Germany).

**Immunofluorescence**—For endogenous detection and overexpression experiments (24 h post-transfection), GnRH cells were plated (15,000/well) on coverslips, and immunofluorescence experiments with FITC-FLAG (1:200) and HDAC6 (1:200) were carried out as described (25). Immunofluorescence for HDAC9 (1:200) was performed as described earlier (15). Coverslips were mounted with prolonged gold containing DAPI (Invitrogen) and observed under confocal microscope (Olympus FV1000 FCS/RCIS, Tokyo, Japan).

**Nuclear and Cytoplasmic Fractionation**—A NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific) was used for nuclear and cytoplasmic extraction from NLT and GT1-7 cells with a modified protocol. For overexpression experiments, cells were harvested 48-h post-transfection and used for fractionation studies.

**Statistical Analysis**—Statistical analyses were performed using GraphPad Software (La Jolla, CA). Data are represented as the mean ± S.E., and statistical differences was analyzed using Student’s t test for two groups and one way analysis of variance with the Bonferroni post hoc test among multiple groups with p < 0.05 considered significant.

**Results**

**Differential Expression of Hdac9 in GnRH Neuronal Cell Lines**—DNA microarray of gene expression profiles in NLT compared with GT1-7 cells were used to determine differences in the transcript levels of different classes of HDACs using bioinformatics tools including Gene Spring software and Ingenuity Pathway Analysis (21). Analysis revealed a consistent increase in the transcript levels for all classes of HDACs (except HDAC7a) in the GT1-7 cells compared with the less differentiated NLT neuronal cells (Fig. 1A). Higher levels of expression for the Class II HDACs, especially Hdac9 (6.7-fold), were observed.

**Increased HDAC9 Expression in GT1-7 Cells**—Differential expression of Hdac9 in GnRH cell lines at the mRNA level was confirmed using qPCR. Increased expression of Hdac9 (4.1-fold, p = 0.0005) (Fig. 1B) along with variable expression of other Class Ila Hdac members including Hdac4 (1.5-fold, p = 0.74) and Hdac5 (1.3-fold, p = 0.08) (data not shown) was seen in GT1-7 cells compared with NLT cells. Hdac7 mRNA levels were not detected in the GnRH neuronal cell lines (data not shown). Increased HDAC9 protein levels were confirmed by immunoblot (4.5-fold, p = 0.03) (Fig. 1C). Immunofluorescence demonstrated that endogenous HDAC9 was predominately localized in the nucleus with a slight localization in the cytoplasmic compartment in the differentiated GT1-7 cells (Fig. 1D). Endogenous localization of HDAC9 was further confirmed using fractionation studies. Endogenous HDAC9 was highly expressed in the nuclear extracts compared with the cytoplasmic extracts in both GT1-7 and NLT cells. Whole cell extracts reveal the difference in HDAC9 expression between both these cell lines. Lamin and β-tubulin were used as nuclear and cytoplasmic markers, respectively (Fig. 1E). Because HDACs are thought to have their major function as deacetylases, we next examined the neuronal-specific HDAC activity levels.

**HDAC9 Activity Is Up-regulated in GT1-7 Cells**—Class-specific HDAC activity was measured in NLT and GT1-7 cells using acetylated class-specific substrates. Acetylated class-specific HDAC substrates, once deacetylated by their respective HDACs, become susceptible to cleavage by trypsin, which releases the fluorophore 7-amino-4-methylcoumarin. Consistent with the observed differences in gene expression, Class I HDAC activity was increased by 1.7-fold in GT1-7 cells (p < 0.05; data not shown). The overall activity in an assay that measures Class Ila HDACs (4, 5, 7, and 9) was not different between the GnRH neuronal cells (Fig. 1F). Because there are no HDAC9-specific substrates available, GT1-7 and NLT cell lysates were immunoprecipitated with a HDAC9-specific antibody (Fig. 1G) and the Class Ila HDAC activity assay was repeated. Fig. 1H shows the increased activity of lysates enriched for HDAC9 (1.5-fold, p = 0.05) in the differentiated GT1-7 cells compared with NLT cells, reflective of the differential expression at the mRNA and protein levels. Together these data suggest that HDAC9 expression and activity increase in models of GnRH neuronal development.

**Pro-survival Role of HDAC9 in GnRH Neuronal Cells**—To explore potential functional roles, HDAC9 was introduced in the less differentiated NLT cells to create a model of GnRH neuronal development (top panel in Fig. 2A), and conversely endogenous HDAC9 was silenced in the more differentiated GT1-7 cells (Top panel in Fig. 2C). Each transfectant was tested in assays of cell survival. Vector control and NLT-HDAC9 cells were exposed to serum deprivation as a model of growth factor...
withdrawal to trigger cell death. NLT-HDAC9 cells showed decreased Hoechst staining of condensed nuclei (0.5-fold, \( p = 0.04 \), Fig. 2A) and reduced caspase-3 cleavage (0.6 \textit{versus} 1.2-fold) (Fig. 2B) in response to serum deprivation compared with vector controls. These data suggested that HDAC9 drives survival of GnRH cell lines. In support of this hypothesis, silencing of endogenous HDAC9 in GT1-7 cells (89% silencing by qPCR; data not shown) resulted in a 4.1-fold (\( p = 0.01 \)) increase in apoptotic cells as assessed by Hoechst staining compared with scramble controls (Fig. 2C). Similarly, silencing of HDAC9 in GT1-7 neurons reversed the protective effects of HDAC9 as assessed by caspase-3 cleavage (2.4-fold, \( p = 0.05 \)) (Fig. 2D).

To determine if silencing of HDAC9 altered the levels of other Class I (\textit{Hdac} 1, 2, 3, and 8) or Class IIa \textit{Hdac} (-4, -5 and
Novel Interaction of HDAC6 and HDAC9 in GnRH Neurons

-7), qPCRs were performed. Similar levels of Hdac1, Hdac2, Hdac3, Hdac8, Hdac4, and Hdac5 were observed after silencing of Hdac9, showing selective inhibition of Hdac9 (data not shown). Hdac7 was not detected in the cell lines. Together these data suggest that HDAC9 may play a role in promoting GnRH neuron survival.

HDAC9 Acts as a Brake on GnRH Neuronal Cell Movement—
To assess the role of HDAC9 in cell movement, migration assays were performed in modified Boyden chamber (23). Overexpression of HDAC9 in NLT neuronal cells resulted in a dramatic inhibition of neuronal cell movement toward serum (6.3-fold, \( p = 0.04 \)) compared with control cells (Fig. 2E). Conversely, silencing of endogenous HDAC9 (86%) in GT1-7 cells resulted in the induction of movement of GT1-7 neuronal cells, which are usually not motile (26) (1.7-fold, \( p = 0.007 \); Fig. 2F).

These results support the hypothesis that induction of HDAC9 during development may play a role in cessation of GnRH neuronal migration.

Migration and Not Survival Effects of HDAC9 Are Dependent on Nuclear and Cytoplasmic Localizations—
To ask if the effects of HDAC9 to ensure cell survival or halt movement was dependent on a nuclear or cytoplasmic location of the protein and determine which domain of the protein mediated these effects, wild type (WT) and truncated HDAC9 (N terminus 1–636, C terminus 637–1069) constructs were tested in the functional assays. Immunohistochemistry revealed that the full-length protein when expressed in NLT cells was localized predominantly in the nuclear compartment with some cytoplasmic expression, similar to endogenous levels in GT1-7 neurons (see Fig. 3A). The HDAC-N truncated mutant (consisting of the regulatory domain and nuclear localization sequences) was specifically targeted to the nucleus, and the HDAC9-C mutant (consisting of the HDAC catalytic domain with a nuclear exclusion sequence) was expressed exclusively in the cytoplasm (Fig. 3A). WT and N- and C-HDAC9 truncated constructs were expressed at equivalent levels in the NLT cells by immunoblot (data not shown). Wild type and truncated HDAC9 proteins were then tested for their effects on cell survival in response to serum starvation-induced apoptosis, both N- and C-terminal truncated HDAC9 constructs, similar to WT HDAC9, showed decreased rates of apoptosis as compared with vector control (73 and 71%, respectively Fig. 3B) and cell migration (Fig. 3C). In response to serum starvation-induced apoptosis, both N- and C-terminal truncated HDAC9 constructs, similar to WT HDAC9, showed decreased rates of apoptosis as compared with vector control (73 and 71%, respectively Fig. 3B) and cell migration (Fig. 3C). However, this function was lost with nuclear-targeted
HDAC9 mutant (24%, p = not significant), suggesting that a cytoplasmic localization is critical for the effects of HDAC9 to inhibit cell movement. Overexpression of N-HDAC9 and C-HDAC9 confirmed their expression in the nuclear extracts and cytoplasmic extracts, respectively, in NLT cells. LAMIN and β-tubulin were used as nuclear and cytoplasmic markers (Fig. 3D).

Nuclear Localization of HDAC9 Leads to Loss of Its Effect on Cell Migration—Additional mutants were then analyzed to ask if the effects of HDAC9 to halt cell movement were dependent on a nuclear to cytoplasmic shuttling function (inset in Fig. 3E). Immunohistochemistry confirmed that the HDAC9 mutant containing a two-amino acid mutation that blocks cytoplasmic translocation (S218A/S448A) showed exclusive nuclear localization (Fig. 3E). In contrast to WT HDAC9, nuclear-localized HDAC9 did not inhibit cell movement (1.4-fold, p = 0.0005 increase compared with controls) in comparison to the inhibitory effects on movement exhibited by the WT mHDAC9 (0.5-fold, p = 0.002) (Fig. 3F). Together these data confirm that cytoplasmic HDAC9 contributes to repression of GnRH neuronal cell movement.

Class IIb HDAC6 Is a Novel Interacting Partner of HDAC9—Little is known about the cytoplasmic partners of HDAC9. Because the Class IIb HDAC6 expression was also increased in GT1-7 cells in the microarray and is a predominantly cytoplasmic protein, experiments determined whether it was a partner of HDAC9. Anti-HDAC6 and anti-HDAC9 antibodies were used to immunoprecipitate HDAC9 or HDAC6 protein. GT1-7 lysates were used as an input control. HDAC9 and HDAC6 were detected at 140 and 132 kDa in immunoprecipitated HDAC6 (Fig. 4A) and HDAC9 (Fig. 4B) lysates, respectively. The interaction of HDAC9 and HDAC6 was specific, as non-immune rabbit IgG precipitated neither HDAC6 nor HDAC9. Immunoprecipitation of HDAC9 revealed no interaction with other members of Class IIa HDACs (HDAC4, HDAC5, and HDAC7) (data not shown). Hdac10, another member of Class IIb, showed higher mRNA levels in NLT rather than GT1-7 neuronal cells, similar to those seen at the transcript levels (data not shown), and did not interact with HDAC9 (data not shown). Together, these data indicate a class member-specific interaction of Class IIa, HDAC9, with Class IIb HDAC6 in GnRH neuronal cells.
Cytoplasmic-localized HDAC6 Is Up-regulated in GT1-7 Cells—Transcript levels of \( \text{Hdac6} \) were up-regulated in GT1-7 cells (6.4-fold) compared with NLT cells (Fig. 4C). Differential transcript expression of \( \text{Hdac6} \) at the mRNA level was confirmed by semiquantitative qPCR (4.3-fold, \( p < 0.05 \); Fig. 4D). HDac6 protein (4.2-fold, \( p = 0.05 \)) was also higher in GT1-7 compared with NLT cells as assessed by immunoblotting (Fig. 4E). Immunocytochemical localization of endogenous HDAC6 confirmed its cytoplasmic localization (Fig. 4F). Endogenous localization of HDAC6 was further confirmed using fractionation studies. HDAC6 was exclusively localized in the cytoplasmic extracts and absent in the nuclear extracts in both NLT and GT1-7 cells. Whole cell extracts reveal the difference in HDAC6 expression between both cell lines (Fig. 4G). Lamin and \( \beta \)-tubulin were used as nuclear and cytoplasmic markers (Fig. 1E). Importantly, HDAC6 activity was also significantly increased (5.4-fold, \( p = 0.005 \)) in GT1-7 compared with NLT neuronal cells, thus supporting the functional importance of cytoplasmic HDAC6 (Fig. 4H).

**HDAC6 Interacts with HDAC9 via Its Second Catalytic Domain**—NLT cells co-expressing FLAG-HDAC9 and HA-HDAC6 were immunoprecipitated with FLAG. HA-tagged HDAC6 was detected in the IP FLAG-HDAC9 lanes, suggesting a specific HDAC6 and HDAC9 interaction in the cell lines (Fig. 4I). IgG control was used as a negative control. To ask which domains of HDAC6 interact with HDAC9, co-immunoprecipitation experiments were performed in NLT cells. FLAG-tagged full-length HDAC6 and truncated HDAC6 constructs containing only the first catalytic domain (1–503) or a construct with both catalytic domains (1–840) were co-expressed with HA-HDAC9 in NLT cells. Lysates were immune-precipitated with a FLAG-specific antibody and immunoblotted with an anti-HA and anti-FLAG antibody (Fig. 5A). HA-HDAC9 was co-precipitated in lysates expressing the FLAG-WT-HDAC6 and truncated FLAG-HDAC6 (1–840) construct but not with the truncated HDAC6 construct containing a single catalytic domain (1–503). To demonstrate that FLAG antibody has no affinity for HA, NLT cells co-expressing FLAG-vector and FLAG-HDAC9 with HA-HDAC6 were immunoprecipitated using anti-FLAG antibody and probed with HA. HA and FLAG was detected only in the HA-HDAC9 lane and not in the vector lane (data not shown). These findings suggest that HDAC6 interacts directly with HDAC9 via its second catalytic domain.

To further elucidate the exact role of the second catalytic domain, WT-HDAC6 and HDAC6 single and double catalytic domain mutants were generated with point mutations in either
or both of the HDAC domains. In NLT cells, low amounts of endogenous HDAC6 were detected across all input and IP lanes (data not shown). Exogenous HDAC6 in both the input and IP lanes was present only in the vector/WT HDAC6- and WT HDAC6-overexpressing lanes and was not in only vector and WT HDAC9-overexpressing lysates, thus confirming overexpression of exogenous HDAC6 and its immunoprecipitation in NLT cells. HDAC9 was detected in all the HDAC6 IP lanes, confirming our earlier observation of interaction between HDAC6 and HDAC9 (Fig. 5B). Overexpression of WT HDAC6 and catalytic domain mutants (in variable amounts) was detected in all the input and IP HDAC6 lanes (Fig. 5B). HDAC9 was identified in immunoprecipitated WT HDAC6/WT HDAC9 and IP mtHDAC6 (DD1)/WT HDAC9; however, this interaction was disrupted in the immunoprecipitation of mtHDAC6 (DD2)/WT HDAC9 lanes (Fig. 5B). To confirm the specificity of the interaction of HDAC6 and HDAC9 via the second catalytic domain of HDAC6, a double catalytic (DC) HDAC6 mutant (H216A/H611A) was co-expressed with WT HDAC9 in NLT cells. HDAC9 was detected using IP of HDAC6 in NLT cells co-expressing WT HDAC6 and WT HDAC9 (Fig. 5C) but lost when WT HDAC9 was co-expressed with the DC-HDAC6 mutant (Fig. 5C). Similarly, HA-HDAC9 was immunoprecipitated with FLAG-HDAC6 but not with the FLAG-DC-HDAC6 mutant using FLAG immunoprecipitation (data not shown).

Additional studies demonstrated that treatment of GT1-7 cells with Tubastatin A (1 μM) lysates immunoprecipitated with anti-HDAC6 failed to show interaction when immunoblotted with HDAC9. IgG was used as a negative control.

or both of the HDAC domains. In NLT cells, low amounts of endogenous HDAC6 were detected across all input and IP lanes (data not shown). Exogenous HDAC6 in both the input and IP lanes was present only in the vector/WT HDAC6- and WT HDAC6-overexpressing lanes and was not in only vector and WT HDAC9-overexpressing lysates, thus confirming overexpression of exogenous HDAC6 and its immunoprecipitation in NLT cells. HDAC9 was detected in all the HDAC6 IP lanes, confirming our earlier observation of interaction between HDAC6 and HDAC9 (Fig. 5B). Overexpression of WT HDAC6 and catalytic domain mutants (in variable amounts) was detected in all the input and IP HDAC6 lanes (Fig. 5B). HDAC9 was identified in immunoprecipitated WT HDAC6/WT HDAC9 and IP mtHDAC6 (DD1)/WT HDAC9; however, this interaction was disrupted in the immunoprecipitation of mtHDAC6 (DD2)/WT HDAC9 lanes (Fig. 5B). To confirm the specificity of the interaction of HDAC6 and HDAC9 via the second catalytic domain of HDAC6, a double catalytic (DC) HDAC6 mutant (H216A/H611A) was co-expressed with WT HDAC9 in NLT cells. HDAC9 was detected using IP of HDAC6 in NLT cells co-expressing WT HDAC6 and WT HDAC9 (Fig. 5C) but lost when WT HDAC9 was co-expressed with the DC-HDAC6 mutant (Fig. 5C). Similarly, HA-HDAC9 was immunoprecipitated with FLAG-HDAC6 but not with the FLAG-DC-HDAC6 mutant using FLAG immunoprecipitation (data not shown). Additional studies demonstrated that treatment of GT1-7 cells with Tubastatin A (1 μM), a known specific catalytic inhibitor of HDAC6 that targets its second catalytic domain, abolished the HDAC9 and HDAC6 interaction (Fig. 5C). Tubulin acetylation was increased after treatment with Tubastatin, indicating the effectiveness of HDAC6 inhibition (data not shown). Together, these data confirm that the interaction of Class IIA HDAC9 with Class Ib HDAC6 occurs via the second catalytic domain of HDAC6.
Interaction of HDAC6 and HDAC9 Controls Cell Survival via the BAX/BCL2 Pathway—The functional role of HDAC6 in cell survival was then tested by silencing it alone or in combination with HDAC9 in GT1-7 cells. HDac6 and Hdac9 and combined silencing showed significant knockdown of HDac6 and HDac9 in GT1-7 cells as seen by real-time PCR (Fig. 6, A and B). Protein levels of knockdown of HDAC6, HDAC9, and HDAC6 and -9 together are shown in Fig. 6C. Cleaved Caspase-3 levels were assessed as an index of cell death. Silencing of HDAC6, HDAC9, or both augmented caspase-3 cleavage (3.4-, 4.4-, and 7.1-fold, respectively, \(p < 0.05\), Fig. 6D), suggesting that HDAC6 and -9 have additive effects to promote GnRH neuronal cell survival. To further elucidate the downstream effectors of HDAC9 and -6 that can promote cell survival, expression of several of the pro and anti-apoptotic pathway molecules were assessed. Silencing of either HDAC6 or HDAC9 alone slightly increased the expression of BAX, a pro-apoptotic marker by (1.6-fold, \(p = 0.48\); 1.3-fold, \(p = 0.65\)). However, this induction was significant only when both were silenced (3.9-fold, \(p = 0.02\)). Conversely, silencing of HDAC6 inhibited the anti-apoptotic marker BCL2 by (3.7-fold, \(p = 0.06\)) and showed a significant decrease only in silencing HDAC9 (9.3-fold, \(p = 0.04\)) alone and in combination 35.6-fold (\(p = 0.04\)) compared with scramble controls (Fig. 6E). Thus HDAC9/6 interaction regulates cell survival via modulating BAX and BCL2, the pro- and anti-apoptotic proteins in the death pathway.

Interaction of HDAC6 and HDAC9 Affects Migration in GnRH Neuronal Cells—To evaluate the combinatorial effects of HDAC6 and HDAC9 on the control of cell movement, modified Boyden chamber assays were performed. Silencing of either HDAC6 or HDAC9 (Fig. 7A) induced GT1-7 cell movement compared with controls (2.6- and 2.3-fold, respectively, \(p < 0.05\)) and together resulted in an additive effect to promote GT1-7 GnRH neuronal movement (5-fold, \(p < 0.05\), Fig. 7A). Together, these in vitro data raise the novel hypothesis that Class IIa and Class IIb HDACs may play a role in the timing of cessation of GnRH neuronal migration in vivo.

To further elucidate the mechanism of HDAC6 and HDAC9 interaction to alter cell movement, levels of acetylated tubulin were measured as an index of cell motility. In NLT cells, overexpression of HDAC6 reduced acetylated \(\alpha\)-tubulin levels compared with vector control (56.6%). However, overexpression of HDAC6 and HDAC9 together failed to reduce this acetylation status, suggesting that HDAC6 interaction with HDAC9 abrogates the acetylation of \(\alpha\)-tubulin, thus leading to impairment in migration (Fig. 7B). Conversely, in GT1-7 cells, silencing of HDAC9 resulted in reduced acetylated tubulin (59.4%). In contrast, silencing of HDAC6 resulted in higher levels of acetylated tubulin. However, silencing of both HDAC9 and HDAC6 blocked the induction of acetylation that was seen with silencing HDAC6 alone (Fig. 7B). These data suggest that the ability of HDAC6 to deacetylate tubulin to induce neuronal migration is lost due to its interaction with HDAC9 in GT1-7 cells, result-
Novel Interaction of HDAC6 and HDAC9 in GnRH Neurons

Recently, Kurian and Terasawa (4) demonstrated that localizing in the hypothalamus has largely remained unexplored. Appropriate development and targeting of GnRH neurons is a complex process that involves precise controls on their survival and migration that can affect ultimately sexual maturation and reproductive competency (1, 22, 27). Cell adhesion molecules, neurotransmitters, growth factors, G-protein receptors, and transcription factors contribute to GnRH neuronal development (1). Additional mechanisms that govern the success of neuronal targeting remain to be elucidated. The process wherein GnRH neurons stop their migration to appropriately localize in the hypothalamus has largely remained unexplored. Recently, Kurian and Terasawa (4) demonstrated that in vitro neuronal maturation of GnRH gene expression coincides with developmental changes in DNA demethylation in post-mitotic neurons, drawing attention to the relatively unexplored field of epigenetic regulation in GnRH neurons.

The roles of HDACs in reproductive and neuronal development are under active investigation. Using HDAC inhibitors as a tool to modulate HDAC function, investigators have shown their role as nuclear transcriptional repressors critical for spermatogenesis (29) to repress FSHβ and LHβ gene expression in immature pituitary gonadotropes (30) and to influence GnRH gene expression (31), thus emphasizing their critical roles in the hypothalamic-pituitary-gonadal axis. Our studies identify Class II HDACs as additional migratory and survival cues in GnRH neuron cells and suggest potential roles for these HDACs in GnRH neurons in vivo.

Our observation of a global activation of all classes of HDACs in the cell models of GnRH neuronal differentiation is in contrast to other systems where many HDACs act only as transcriptional “brakes” and are turned off with development. Undifferentiated myocytes, adipocytes, and cholinergic neurons demonstrated high expression of HDAC9, whereas relatively lower levels were detected in the differentiated state (12, 32, 33). For example, Mitf/Hdarp, an Hdac9 splice variant, represses MEF2 targets by recruiting Class I HDACs but then is down-regulated with muscle denervation to allow up-regulation of the acetylcholine receptor subunits (33). In cholinergic neurons, HDAC9 is also a negative regulator of differentiation and is inhibited to allow activation of the choline acetyltransferase gene during neuronal development (32). However, in the current experiments, most HDACs and Class II HDACs, in particular, were up-regulated in the more differentiated cell line, suggesting a cell-specific developmental program. HDAC9 was highly expressed in the dissected hypothalamus from adult mice, suggesting it could influence the hypothalamic locus of the reproductive axis in vivo.

Prior work has shown that Class IIa HDACs (HDAC4, -5, and -7) compensate for each other when one is silenced to promote responses to glucagon and insulin signaling in the liver (14). HDAC9 can interact with nuclear co-repressor (N-CoR) as well as HDAC family members (HDAC1, HDAC3, and HDAC4) involved in hematological malignancies (16). In the current study, HDAC9 did not co-immunoprecipitate with any other HDAC family members had not been previously reported. Our study is the first to demonstrate this novel interaction between a Class IIa and IIb HDAC family member.

Discussion

Appropriate development and targeting of GnRH neurons is a complex process that involves precise controls on their survival and migration that can affect ultimately sexual maturation and reproductive competency (1, 22, 27). Cell adhesion molecules, neurotransmitters, growth factors, G-protein receptors, and transcription factors contribute to GnRH neuronal development (1). Additional mechanisms that govern the success of neuronal targeting remain to be elucidated. The process wherein GnRH neurons stop their migration to appropriately localize in the hypothalamus has largely remained unexplored. Recently, Kurian and Terasawa (4) demonstrated that in vitro neuronal maturation of GnRH gene expression coincides with

![FIGURE 7. HDAC9 and HDAC6 modulates migration. Panel A, the bar graph depicts amplified basal movement in both HDAC6 and HDAC9 silenced GT-1–7 cells with augmented effects on basal cell movement after silencing both HDAC6 and HDAC9 as assessed by modified Boyden chamber assay. \( p < 0.05, n = 3 \). Panel B, overexpression (top panel) or silencing (bottom panel) of HDAC6 and HDAC9 together impede acetylation of α-tubulin in NLT and GT1-7 cells, respectively, with total α-tubulin as a loading control. Panel C, schematic illustration of HDAC9 and HDAC6 interaction and their role in modulating survival and movement of GT1-7 neuronal cells.](image-url)
Isotype effects of HDACs have been previously shown to have additive, independent effects on T cell function. HDAC6 and HDAC9 can each modulate Foxp3 expression and hyper-acetylation Hsp90, thus controlling regulatory T cell function; however, no analysis was performed of any interaction between HDACs (41). HDAC6 and SIRT2 can positively regulate acetylation of K-ras, an oncogene in cancer cells, thus suggesting a possible combined therapeutic target utilizing interactions between different classes of HDACs (40). The current studies suggest a paradigm-shifting model where Class IIa HDAC9 and Class IIb HDAC6 directly interact to modulate cell survival and halt cell movement through unique cytoplasmic and nuclear actions (Fig. 7C). In other systems calcium/calmodulin and protein phosphatases modulate shuttling of Class IIa HDACs. The specific phosphatases reported to regulate Class IIa HDACs include PP1B and MYPT1 as HDAC7 partners in the immune system and PP2A with HDAC7 and -4 in muscle and bone cells (11). Also, recent library screens suggest that Class II HDACs may interact with other cell-specific kinases including AMPK, MASK2, and Mirk/SIK1, -2, and -3 and checkpoint kinases (34). Further studies are needed to determine whether there are GnRH neuronal cell-specific mechanisms that modulate nuclear-cytoplasmic shuttling of HDAC9.

Interaction with HDAC9 in GnRH neuronal cell lines was dependent on a direct interaction with the second catalytic domain of HDAC6. Among the HDACs, the first and second catalytic domains of HDAC6 show a higher (50 and 55%) similarity to the catalytic domain of HDAC9 (42). HDAC9 did not interact with the other Class IIb HDAC, HDAC10, confirming the specificity of the HDAC9 and -6 interaction. Because HDAC10 lacks a functional second catalytic domain compared with HDAC6, the lack of interaction between HDAC9 and HDAC10 supports the hypothesis that interaction of HDAC9 and HDAC6 is dependent on the second catalytic domain.

In GnRH neuronal cells, HD40

References

1. Wierman, M. E., Kiseljak-Vassiliades, K., and Tobet, S. (2011) Gonadotropin-releasing hormone (GnRH) neuron migration: initiation, maintenance and cessation as critical steps to ensure normal reproductive function. Front. Neuroendocrinol. 32, 43–52
2. Schwarting, R. A., Wierman, M. E., and Tobet, S. A. (2007) Gonadotropin-releasing hormone neuronal migration. Semin. Reprod. Med. 25, 305–312
3. Tobet, S. A., and Schwarting, G. A. (2006) Minireview: recent progress in gonadotropin-releasing hormone neuronal migration. Endocrinology 147, 1159–1165
4. Kurian, J. R., and Terasawa, E. (2013) Epigenetic control of gonadotropin-releasing hormone neurons. Front. Endocrinol. (Lausanne) 4, 61
5. Terasawa, E., Kurian, J. R., Guerriero, K. A., Keneally, B. P., Hutz, E. D., and Keen, K. L. (2010) Recent discoveries on the control of gonadotropin-releasing hormone neurons in nonhuman primates. J. Neuroendocrinol. 22, 630–638
6. Witt, O., Deubzer, H. E., Milde, T., and Oehme, I. (2009) HDAC family: what are the cancer relevant targets? Cancer Lett. 277, 8–21
7. Martin, M., Kettmann, R., and Dequiedt, F. (2007) Class IIa histone deacetylases: regulating the regulators. Oncogene 26, 5450–5467
8. de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S., and van Kuijlenburg, A. B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 370, 737–749

Acknowledgments—We thank Todd Horn for performing the HDAC catalytic activity assays. Imaging experiments were performed in the University of Colorado Anschutz Medical Campus Advance Light Microscopy Core supported in part by National Center for Advancing Translational Sciences, National Institutes of Health Colorado Clinical and Translational Sciences Institute Grant UL1 TR001082.
Novel Interaction of HDAC6 and HDAC9 in GnRH Neurons

9. Haberland, M., Montgomery, R. L., and Olson, E. N. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. 10, 32–42

10. Zhou, X., Marks, P. A., Rijkind, R. A., and Richon, V. M. (2001) Cloning and characterization of a histone deacetylase, HDAC9. Proc. Natl. Acad. Sci. U.S.A. 98, 10572–10577

11. Bertos, N. R., Wang, A. H., and Yang, X. J. (2008) Histone acetyltransferases: structure, function, and regulation. Biochem. Cell Biol. 79, 243–252

12. Chatterjee, T. K., Idelman, G., Blanko, V., Blomkalns, A. L., Piegro, M. G., Jr., Weintraub, D. S., Kumar, S., Rajeshker, S., Manka, D., Rudich, S. M., Tang, Y., Hui, D. Y., Basset-Duby, R., Olson, E. N., Lingrel, J. B., Ho, S. M., and Weintraub, N. L. (2011) Histone deacetylase 9 is a negative regulator of adipogetic differentiatation. J. Biol. Chem. 286, 27836–27845

13. Zhang, C. L., McKinsey, T. A., Chang, S., Santos, C. L., Hill, J. A., and Olson, E. N. (2002) Class II histone deacetylases act as signal-responsive regulators of cardiac hypertrophy. Cell 110, 479–488

14. Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol. Cell. Biol. 24, 8467–8476

15. Sugo, N., Oshiro, H., Takeamura, M., Kobayashi, T., Kohno, Y., Usaka, N., Song, W. I., and Yamamoto, N. (2010) Nucleo-cytoplasmic translocation of HDAC9 regulates gene expression and dendritic growth in developing cortical neurons. Eur. J. Neurosci. 31, 1521–1532

16. Petrie, K., Guizide, F., Howell, L., Healy, L., Waxman, S., Greaves, M., and Zelent, A. (2003) The histone deacetylase 9 gene encodes multiple protein isoforms. J. Biol. Chem. 278, 16059–16072

17. Valenzuela-Fernández, A., Cabrero, J. R., Serrador, J. M., and Sánchez-Madrid, F. (2008) HDAC6: a key regulator of cytoskeleton, cell migration, and cell-cell interactions. Trends Cell Biol. 18, 291–297

18. Radovick, S., Wray, S., Lee, E., Nicolas, D. K., Nakayama, Y., Weintraub, B. D., Westphal, H., Cutler, G. B., Jr., and Woudiford, F. E. (1991) Mutagenic assay of gonadotropin-releasing hormone neurons in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 88, 3402–3406

19. Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padula, C. A., Roberts, J. L., and Weiner, R. I. (1990) Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5, 1–10

20. Salian-Mehta, S., Xu, M., Knox, A. J., Plummer, L., Slavov, D., Taylor, M., Bevers, S., Hodges, R. S., Crowley, W. F., Jr., and Wierman, E. M. (2014) Functional consequences of AXL sequence variants in hypogonadotropic hypogonadism. J. Clin. Endocrinol. Metab. 99, 1452–1460

21. Pierce, A., Bliesner, B., Xu, M., Nielsen-Petersen, S., Lemke, G., Tobet, S., and Wierman, M. E. (2008) Axil and Tyro3 modulate female reproduction by regulating gonadotropin-releasing hormone neuron survival and migration. Mol. Endocrinol. 22, 2481–2495

22. Allen, J. L., Passerini, F., Zanetti, J. A., and Hunter, T. (2002) Histone deacetylase-related gene and protein. U. S. Patent WO2002030970A2

23. Alonso, S., Sanchez-Madrid, F., and Schaffner, L. (2005) Histone deacetylase 9 couples neuronal activity to muscle cholinat acetylation and gene expression. Nat. Neurosci. 8, 313–321

24. Gao, Y. S., Hubbert, C. C., Lu, J., Lee, Y. S., Lee, J. Y., and Yao, T. P. (2007) Histone deacetylase 6 regulates growth factor-induced actin remodeling and endocytosis. Mol. Cell. Biol. 27, 8637–8647

25. Li, Y., Shin, D., and Kwon, S. H. (2013) Histone deacetylase 6 plays a role as a distinct regulator of diverse cellular processes. FIBBS / 280, 775–793

26. Zhang, Y., Li, N., Caron, C., Matthis, G., Hess, D., Knochlin, S., and Mathias, P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. EMBO J. 22, 1168–1175

27. Zhang, X., Yuan, Z., Zhang, Y., Song, S., Salas-Burgos, A., Koomen, J., Olashaw, N., Parsons, J. T., Yang, X. J., Dent, S. R., Yao, T. P., Lane, W. S., and Seto, E. (2007) HDAC6 modulates cell motility by altering the acetylation level of cortactin. Mol. Cell. 27, 197–213

28. Gao, L., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J. Biol. Chem. 277, 25748–25755

29. Yang, M. H., Laurent, G., Bause, S. A., Spang, R., Germain, N., Haigis, M. C., and Haigis, K. M. (2013) HDAC6 and SIRT2 regulate the acetylation state and oncogenic activity of mutant K-RAS. Mol. Cancer Res. 11, 1072–1077

30. Beier, U. H., Wang, L., Han, R., Akimova, T., Liu, Y., and Hancock, W. W. (2012) Histone deacetylases 6 and 9 and sirtuin-1 control F0x3+/ regulatory T cell function through shared and isoform-specific mechanisms. Sci. Signal. 5, ra45

31. Bhutia, U., Cai, R. L., Cohen, D., and Fischer, D. D. (April 18, 2002) Histone deacetylase-related protein. U. S. Patent WO2002030970A2

32. Insigna, A., Monestiroli, S., Ronzoni, S., Gelmetti, V., Marchesi, F., Viale, A., Altucci, L., Nervi, C., Minucci, S., and Pelicci, P. G. (2005) Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat. Med. 11, 71–76

33. Thangaraju, M., Carswell, K. N., Prasad, P. D., and Ganapathy, V. (2009) Novel Interaction of HDAC6 and HDAC9 in GnRH Neurons

34. Bardai, F. H., Price, V., Zaayman, M., Wang, L., and D’Mello, S. R. (2012) Histone deacetylase-related gene and protein. U. S. Patent WO2002030970A2

35. Insinga, A., Monestiroli, S., Ronzoni, S., Gelmetti, V., Marchesi, F., Viale, A., Altucci, L., Nervi, C., Minucci, S., and Pelicci, P. G. (2005) Inhibitors of histone deacetylases induce tumor-selective apoptosis through activation of the death receptor pathway. Nat. Med. 11, 71–76

36. Changarao, M., Carswell, K. N., Prasad, P. D., and Ganapathy, V. (2009) Colon cancer cells maintain low levels of pyruvate to avoid cell death caused by inhibition of HDAC1/HDAC3. Biochem. J. 417, 379–389

37. Bardin, B. H., Price, V., Zaatman, Y., Wang, L., and D’Mello, S. R. (2012) Histone deacetylase-1 (HDAC1) is a molecular switch between neuronal survival and death. J. Biol. Chem. 287, 35444–35453

38. Yuan, Z., Peng, L., Radhakrishnan, R., and Seto, E. (2010) Histone deacetylase 9 (HDAC9) regulates the functions of the ATDC (TRIM29) protein. J. Biol. Chem. 285, 39329–39338

39. Morrison, B. E., Majdzadeh, N., Zhang, X., Lyles, A., Bassel-Duby, R., Olson, E. N., and D’Mello, S. R. (2006) Neuroprotection by histone deacetylase-related protein. Mol. Cell. Biol. 26, 3530–3564