

# Histone Deacetylase (HDAC) Activity Is Critical for Embryonic Kidney Gene Expression, Growth, and Differentiation<sup>\*[5]</sup>

Received for publication, April 6, 2011, and in revised form, June 27, 2011 Published, JBC Papers in Press, July 21, 2011, DOI 10.1074/jbc.M111.248278

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Histone deacetylases (HDACs) regulate fundamental biological processes such as cellular proliferation, differentiation, and survival via genomic and nongenomic effects. This study examined the importance of HDAC activity in the regulation of gene expression and differentiation of the developing mouse kidney. Class I HDAC1–3 and class II HDAC4, -7, and -9 genes are developmentally regulated. Moreover, HDAC1–3 are highly expressed in nephron precursors. Short term treatment of cultured mouse embryonic kidneys with HDAC inhibitors (HDACi) induced global histone H3 and H4 hyperacetylation and H3K4 hypermethylation. However, genome-wide profiling revealed that the HDAC-regulated transcriptome is restricted and encompasses regulators of the cell cycle, Wnt/ $\beta$ -catenin, TGF- $\beta$ /Smad, and PI3K-AKT pathways. Further analysis demonstrated that base-line expression of key developmental renal regulators, including *Osr1*, *Eya1*, *Pax2/8*, *WT1*, *Gdnf*, *Wnt9b*, *Sfrp1/2*, and *Emx2*, is dependent on intact HDAC activity. Treatment of cultured embryonic kidney cells with HDACi recapitulated these gene expression changes, and chromatin immunoprecipitation assays revealed that HDACi is associated with histone hyperacetylation of *Pax2/Pax8*, *Gdnf*, *Sfrp1*, and *p21*. Gene knockdown studies demonstrated that HDAC1 and HDAC2 play a redundant role in regulation of *Pax2/8* and *Sfrp1* but not *Gdnf*. Long term treatment of embryonic kidneys with HDACi impairs the ureteric bud branching morphogenesis program and provokes growth arrest and apoptosis. We conclude that HDAC activity is critical for normal embryonic kidney homeostasis, and we implicate class I HDACs in the regulation of early nephron gene expression, differentiation, and survival.

Kidney development depends on reciprocal inductive interactions between the metanephric mesenchyme (MM),<sup>4</sup> a spec-

ified region in the caudal intermediate mesoderm, and the ureteric bud (UB), an epithelial outgrowth from the Wolffian (nephric) duct (1–3). Recent years have witnessed significant progress in our understanding of the gene regulatory networks of early kidney development (3–6). For example, the *Osr1/Eya1/Pax2/Six/Sall/WT1/Hoxd11* gene regulatory network specifies the MM and is absolutely required for expression of glia-derived neurotrophic factor (*Gdnf*) (7, 8). *Gdnf*, in turn, is essential for UB outgrowth and subsequent branching (9–11). *Gdnf* acts via activation of a c-Ret/PI3K/ERK-dependent gene network (*Wnt11*, *Spry1*, *Etv4*, *Etv5*, *Cxcr4*, *Myb*, *Met*, and *Mmp14*) in UB tip cells to control the branching morphogenesis program (12–14). Various growth factor/receptor signaling pathways, including FGFs, bone morphogenic proteins, VEGF, semaphorins, hepatocyte growth factor, EGF, among others, share signaling components with the c-Ret pathway and are required for optimal metanephric growth and patterning (15–22).

Following induction of the MM, activation of the Wnt/ $\beta$ -catenin signaling pathway plays a key role in nephrogenesis. Release of Wnt9b from the UB branches triggers a  $\beta$ -catenin-dependent morphogenetic program leading to expression of *Wnt4*, *Fgf8*, and *Pax8* and transition of ventrally located MM cells to epithelial nephron progenitors (pretubular aggregates and renal vesicles) (23, 24). Wnt9b signaling cooperates with Six2, a transcription factor expressed exclusively in the dorsal metanephric cap region to maintain the proliferation or self-renewal of pluripotent MM cells (25–27). Subsequently, the Lim homeodomain transcription factor, Lhx1 (also known as Lim-1), a downstream target of Wnt4/Fgf8 signaling, mediates further maturation to comma- and S-shaped bodies (28–30). Segmentation of the epithelial progenitor to proximal and distal fates is partly accomplished by signaling pathways downstream of Notch and *Irx/Brn-1*, respectively (31–34). The canonical Wnt/ $\beta$ -catenin signaling pathway is also required for UB outgrowth and branching (35, 36).

Histone deacetylases (HDACs) are an evolutionarily conserved group of enzymes that regulate chromatin architecture and gene expression by removing acetyl groups from histone tails. To date, 18 mammalian HDACs have been identified. Based on sequence homology to yeast *hda* genes, they are divided into four classes as follows: class I HDACs (1–3 and 8), class II HDACs (4–7, 9, and 10), class III (Sirtuins 1–7), and class IV (HDAC11) (37–39). The action of HDACs on histone

\* This work was supported, in whole or in part, by National Institutes of Health Grants DK-079886, DK-62550, and DK56264 (to S. S. E.-D.).

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–6.

<sup>1</sup> Supported by a fellowship grant from the National Kidney Foundation.

<sup>2</sup> Supported by Grant 1P20 RR-017659 from Centers of Biomedical Research Excellence.

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<sup>4</sup> The abbreviations used are: MM, metanephric mesenchyme; HDAC, histone deacetylase; HDACi, HDAC inhibitor; UB, ureteric bud; mK4 cells, metanephric kidney cell line; MO, morpholino; TSA, trichostatin A; ISH, *in situ* hybridization.

## Histone Deacetylases in Renal Development

acetylation is counteracted by histone acetyltransferases, e.g. CBP/p300 and GCN5, which acetylate histone tails.

Although the fundamental functions of HDACs in cancer and cardiac and immune disorders are well documented, their role in embryogenesis and organogenesis has just begun to be elucidated (40). HDAC1 and HDAC2 are crucial for regulating cell proliferation and are indispensable for embryo survival (41, 42). HDAC1 and HDAC2 perform redundant yet essential functions in cardiac growth and development, in the pathological responses to increased cardiac load, and differentiation of oligodendrocytes (43, 44). Class II HDACs are important for restricting cell size; HDAC4, -5, and -9 exert antihypertrophic properties in chondrocytes and heart, respectively (45, 46). In addition, HDAC7 null mice are embryonic lethal by E11.0 secondary to loss of vascular integrity (47). The role of HDACs in mammalian kidney development is unknown, but there is evidence that HDACs regulate the progenitor cell population in the pronephric kidney of zebrafish (48).

In this study, we show that several members of class I and II HDACs are developmentally regulated in the kidney. Furthermore, using pharmacological inhibitors of HDACs in *ex vivo* cultured embryonic kidneys, we show that HDAC activity is required for expression of key developmental pathways. Moreover, gene knockdown studies and ChIP assays revealed a redundant role for HDAC1 and HDAC2 in MM cell gene expression. Finally, we show that HDAC activity is necessary for growth and survival of the developing nephron.

### EXPERIMENTAL PROCEDURES

**RT-PCR and Real Time RT-PCR**—Total RNA was isolated from embryonic kidneys or cells using TRIzol reagent (Invitrogen) or RNAqueous-96 automated kit (Ambion, Austin, TX). For semi-quantitative RT-PCR assays, 1  $\mu$ g of total RNA from each sample was reverse-transcribed by SuperScript II RT (Invitrogen) in a 20- $\mu$ l volume. Sequences of PCR primers are shown in Table 1. PCR products were run on 2% agarose gel and digitally analyzed using FluorChem FC2 imaging system (Alpha Innotech, San Leandro, CA). *Gapdh* was used as an internal control for the normalization of gene expression. For TaqMan real time PCR, assays were performed using the One-Step Brilliant<sup>®</sup> quantitative RT-PCR master mix kit (Stratagene) containing 200 nM forward primer, 200 nM reverse primer, and 60 ng of total RNA. Relative levels of mRNA were normalized to  $\beta$ -actin. The following real time primers were purchased from Applied Biosystems: *Pax2* Mm00477972\_m1; *Pax8* Mm00440623\_m1; *Gdnf* Mm00599849\_m1; and *Sfrp1* Mm00489161\_m1.  $\beta$ -Actin primers and probe sequences were as follows: forward 5'-ACGGCCAGGTCACTATTG-3'; reverse 5'-CAAGAAGGAAGGCTGGAAAAG-3'; and probe (5-hexachloro-fluorescein)-CAACGAGCGGTTCCGATGCCC.

**Western Blotting**—Nuclear and cytosolic proteins were extracted separately by using nuclear/cytosol fractionation kit (BioVision, Mountain View, CA), and whole cell proteins were extracted using RIPA buffer (Invitrogen). Histones were isolated by the acid extraction technique. Briefly, tissues were homogenized and lysed in ice-cold Triton Extraction Buffer (TEB/PBS containing 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) and then

**TABLE 1**  
Primers for RT-PCR analysis

Gene	Primers sequences
<i>Gapdh</i>	Sense, 5'-CCCCTGGCCAAGGTCATCCATGACAACCTT-3' Antisense, 5'-GGCCATGAGGTCCACCACCCTGTTGCTGTA-3'
<i>Hdac1</i>	Sense, 5'-CTGTCCGGTATTGTATGGCT-3' Antisense, 5'-CACGAACCTCCACACACTTGG-3'
<i>Hdac2</i>	Sense, 5'-GGCGGCAAGAAGAAAGTGTGC-3' Antisense, 5'-GGCATCATGTAGTCCCTCCAGC-3'
<i>Hdac3</i>	Sense, 5'-TCTGAGGACTACATCGACTCC-3' Antisense, 5'-GTCGCATCATAGAACTCATTG-3'
<i>Hdac4</i>	Sense, 5'-CAGATGGACTTTCTGGCCG-3' Antisense, 5'-CTTGAGCTGCTGCAGCTTC-3'
<i>Hdac5</i>	Sense, 5'-GAAGCACCTCAAGCAGCAGCAGG-3' Antisense, 5'-CACTCTCTTTGCTCTTCTCCTTGT-3'
<i>Hdac6</i>	Sense, 5'-ACGCTGACTACATGTGCTGCT-3' Antisense, 5'-TCTCAACTGATCTCTCCAGG-3'
<i>Hdac7</i>	Sense, 5'-AGCTGGCTGAAGTATCC-3' Antisense, 5'-TCACCATCAGCCTCTGAG-3'
<i>Hdac8</i>	Sense, 5'-AACACGGCTCGATGCTGG-3' Antisense, 5'-CCAGCTGCCACTTGATGC-3'
<i>Hdac9</i>	Sense, 5'-TCAGAGTTTCCATGCGCCTG-3' Antisense, 5'-TGGAGACGTTCCACTGAGGG-3'
<i>P21</i>	Sense, 5'-CACAGGCACCATGTCCAATC-3' Antisense, 5'-GAAATCTGTGAGGCTGGTCT-3'
<i>Pax2</i>	Sense, 5'-TTTGTGAACGGCCGGCCCTA-3' Antisense, 5'-CATTGTCACTGACCTCCG-3'
<i>Pax8</i>	Sense, 5'-ATTCCAGCATTGCCGCTCAC-3' Antisense, 5'-TGGTCCCATCTGTGTGCTTCC-3'
<i>Six2</i>	Sense, 5'-CTCAAGGCGCACTACATC-3' Antisense, 5'-ACTGCCATTGAGCGAGGAAG-3'
<i>Eya1</i>	Sense, 5'-CTAACCAGCCCGCATAGCCG-3' Antisense, 5'-TAGTTTGTGAGGAGGGGTAGG-3'
<i>WT1</i>	Sense, 5'-ACCCAGGCTGCAATAAGAGA-3' Antisense, 5'-GCTGAAGGGCTTTTCACTTG-3'
<i>Gdnf</i>	Sense, 5'-GCCCTTCGCGCTGACCAGGTGAC-3' Antisense, 5'-GATGGTGGCGATAGTGGGATA-3'
<i>FoxD1</i>	Sense, 5'-TGTCAGTGTGGAGAACTTTACTG-3' Antisense, 5'-CTCTACACCTCAAAAAGGGCTTAG-3'
<i>Fgf8</i>	Sense, 5'-ACCAACTCTACAGCCGACCAG-3' Antisense, 5'-GTAGTTGAGGAACCTCGAAGCCGAG-3'
<i>Sfrp1</i>	Sense, 5'-GGTCATCGAGGTTCTTCGGTCTCTAC-3' Antisense, 5'-CGCTTCAGCTCCTTCTTCTTGATG-3'
<i>Sfrp2</i>	Sense, 5'-GCCTCGTCTGCTGTGTAGTC-3' Antisense, 5'-TGTCGTTTGTGCTCCTCATTCTTG-3'
<i>Bmp4</i>	Sense, 5'-TCCTGGTAACCGAATGCTGATG-3' Antisense, 5'-GATGGAACCTCCTCACAGGTTGG-3'
<i>Wnt9b</i>	Sense, 5'-GCAAGTGCACGGTGTGTCA-3' Antisense, 5'-TCCCCTGGAGGCTTAGCGCTTGAGGT-3'
<i>cRet</i>	Sense, 5'-GGCATTAAAGCAGGCTACGGCA-3' Antisense, 5'-GAGGAATAACTGATTGGGAA-3'
<i>Wnt11</i>	Sense, 5'-CTGGCACTGTCCAAGACTCC-3' Antisense, 5'-AGCTCGATGGAGAGCAGT-3'
<i>Spry1</i>	Sense, 5'-TAGGTCAGATCGGGTCATCC-3' Antisense, 5'-CACAGGTATCTGGAGCAGCA-3'
<i>Wnt4</i>	Sense, 5'-ATCGGTGGCAGCATCT-3' Antisense, 5'-CTTCTGCCAGCCTCGT-3'
<i>Lhx1</i>	Sense, 5'-CATCCCCTGACTCTAATTGC-3' Antisense, 5'-CAAAGGCTGCCTTCAACG-3'

centrifuged at 2,000  $\times$  g for 10 min at 4  $^{\circ}$ C. The nuclear pellet was washed once in TEB and resuspended in 0.2 N HCl overnight at 4  $^{\circ}$ C, followed by centrifugation at 2,000  $\times$  g for 10 min at 4  $^{\circ}$ C. The histone-enriched supernatant was collected and stored at -80  $^{\circ}$ C. The following primary antibodies were used: anti-HDAC1-3 antibodies (all rabbit polyclonal, 1:200-1:500, BioVision, Mountain View, CA), anti-acetyl-H3 (1:1000, Upstate Biotechnology, Inc., Lake Placid, NY), anti-acetyl-H4 (1:500, Upstate Biotechnology, Inc.), anti-Pan H3 (1:1000, Upstate Biotechnology, Inc.), and mouse anti- $\beta$ -actin (1:5000, Sigma).

**Immunohistochemistry**—Kidneys were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4  $\mu$ m. Antigen retrieval was accomplished by placing slides in boiling 10 mM sodium citrate, pH 6.0, for 20 min. Immunostaining was per-

formed with the immunoperoxidase technique using the ABC Elite Vectastain kit (Vector Laboratories, Burlingame, CA). The primary antibodies against HDACs were as listed above and used at concentrations ranging from 1:50 to 1:500. For immunofluorescence, the secondary antibodies were Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-rabbit (1:2000, Invitrogen) and anti-mouse FITC (1:200, Sigma).

**Whole Mount Immunofluorescence**—Kidney explants were fixed in 100% methanol for 10 min and then stored in PBS, 0.1% Tween 20 (PBST) at 4 °C. The samples were incubated with the primary antibodies diluted in PBST overnight at 4 °C. The samples were then washed in PBST for 8 h with changing the solution every 1 h. Secondary antibodies were diluted in PBST and placed on samples overnight at 4 °C and washed extensively in PBST. The tissues were mounted on glass slides with Dako fluorescent mounting media (Dako, Carpinteria, CA) and stored at 4 °C until imaging. The primary antibodies used included anti-pan cytokeratin (1:200, Sigma), anti-Pax2 (1:100, Zymed Laboratories Inc.), and anti-WT1 (C-19; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies included Alexa Fluor 350 anti-mouse (1:64, Sigma) and Alexa Fluor 594 anti-rabbit (1:400, Invitrogen). In addition, FITC-conjugated *Lotus tetragonolobus* lectin agglutinin (1:100, Vector Laboratories) was used to label the apical brush border of proximal tubules.

**Metanephric Organ Culture**—Embryonic kidneys were aseptically microdissected from timed-pregnant Hoxb7-GFP<sup>+</sup> mice (49) or CD1 mice and were cultured on polycarbonate transwell filters (0.4- $\mu$ m pore size, Corning Costar, Acton, MA) over medium (DMEM/F-12 + 10% FBS) at 37 °C and 5% CO<sub>2</sub>. Paired metanephroi were treated with the HDAC inhibitor (HDACi) or control drug. Three different HDACi were used at the indicated concentrations (see under “Results”) as follows: trichostatin A (BioVision), Scriptaid (Biomol, Plymouth Meeting, PA), and MS-275 (Sigma). Nullscript, an inactive structural analog of Scriptaid, was used as a control drug for Scriptaid, and DMSO was used as a control for TSA and MS-275.

**Cell Proliferation and Apoptosis Assays**—Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation (Invitrogen). BrdU was added into the culture medium at final concentration of 10  $\mu$ M 2 h before the end of HDACi treatment. Apoptosis was assessed using terminal deoxynucleotidyltransferase biotin-dUTP nick end-labeling (TUNEL) and was carried out using an *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD). The proliferation/apoptosis index (percentage of BrdU-positive or TUNEL-positive cells) was determined in four randomly picked microscopic fields of each kidney section.

**Genome-wide Microarray Analysis**—Microarray analysis was performed according to established protocols (50). The results represent three separate microarray experiments. Total RNA was isolated from CD1 mouse kidney explants, cultured in the presence of Scriptaid (2  $\mu$ g/ml) or its inactive analog Nullscript (2  $\mu$ g/ml) for 6 h, using RNAqueous-96 automated kit (Ambion). The quality of RNA samples was assessed by capillary electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). Fluorescently labeled cRNA was generated from 0.5  $\mu$ g of total RNA in each reaction using the Agilent fluorescent direct label kit and 1.0 mM cyanine 3- or 5-labeled dCTP (PerkinElmer Life Sciences) following the owner’s man-

ual. Cyanine-labeled cDNA from Nullscript-treated kidney sample was mixed with the same amount of reverse-color cyanine-labeled cDNA from Scriptaid-treated kidney sample. Alternatively, cyanine-labeled cDNA from Scriptaid-treated kidney sample was mixed with the same amount of reverse-color cyanine-labeled cDNA from Nullscript-treated kidney sample. Hybridization was performed using the oligonucleotide microarray hybridization and *in situ* hybridization plus kit from Agilent (following the owner’s manual). The labeled cRNA was hybridized to Agilent 44K whole mouse genome oligonucleotide microarray (containing ~41,000 Probes) as described previously (50). The arrays were scanned using a dual-laser DNA microarray scanner (Agilent). The data were then extracted from images by the Feature Extraction software 6.1 (Agilent).

**Data Analysis**—The GeneSpring 7.3 software (Agilent) was used to generate lists of selected genes and for different statistical and visualization methods. An intensity-dependent normalization (known as Lowess normalization) was applied to correct for artifacts caused by nonlinear rates of dye incorporation as well as inconsistencies of the relative fluorescence intensity between some red and green dyes. The differentially expressed genes between the experimental groups and control groups were identified by a *p* value cutoff of 0.05 and a minimum 1.5-fold up- or down-regulation of gene expression. The genes in the gene lists were classified according to their function using the Gene Ontology (GO SLIMS) and BINGO classification systems. Additional analysis of the microarray data were completed using the Ingenuity Pathway Analysis (IPA) software.

**Whole Mount *in Situ* Hybridization**—*In situ* hybridization (ISH) was performed using digoxigenin-labeled antisense probes. In brief, cultured metanephroi from HDACi-treated and control groups were processed simultaneously and fixed in 4% paraformaldehyde, dehydrated in methanol, and stored at -20 °C prior to staining. After rehydration in 0.1% Tween in PBS, the samples were digested with proteinase K, and then refixed in 4% paraformaldehyde, 0.2% glutaraldehyde followed by three washes with PBS. After a 3-h incubation in hybridization solution, the explants were hybridized with the digoxigenin-labeled antisense probes (~1  $\mu$ g of probe/vial) overnight at 65 °C. The next day, the samples were sequentially washed with Hybridization Solution, 2 $\times$  SSC mixture, pH 4.5, 2 $\times$  SSC, pH 7.0, 0.1% CHAPS, maleic acid buffer, and PBS at room temperature. The explants were incubated with preblocked antibody (1:10,000, anti-Dig alkaline phosphatase, Roche Applied Science) at 4 °C overnight. The following day, after sequential washes of 0.1% BSA in PBS, PBS, and AP1 buffer at room temperature, the samples were stained by BM Purple (Roche Applied Science) at 4 °C. When the desired level of staining was reached, the reaction was stopped by two washes of Stop Solution for 15 min each. The samples were then dehydrated in methanol and stored at 4 °C. The experimental and control samples were put in the same reaction vessel to allow for proper comparison.

**Knockdown HDAC1 and HDAC2 in mK4 Cells Using Morpholino Oligonucleotides (MOs)**—Antisense morpholino oligonucleotides to HDAC1 and HDAC2 and standard control mor-

## Histone Deacetylases in Renal Development

pholino were obtained from Gene Tools, LLC (Philomath, OR). The *HDAC1-MO* is a translation blocker 5'-TGC CCT GAG TCT GCG CCA TCT TGCT-3'. *HDAC2-MO1* is a translation blocker 5'-TGC CGC CTC CTT GAC TGT ACG CCAT-3'. *HDAC2-MO2* is a splice-blocking morpholino 5'-ACA CAG TGC CAA CTA GTA CTT ACAT-3'. *HDAC2-MO1* and *MO2* were used together to maximize inhibition of HDAC2 expression. Endo-Porter was used to deliver the morpholinos. mK4 cells were incubated with HDAC1-MO (10  $\mu$ M) and/or HDAC2-MO1/2 (10  $\mu$ M of *tbHDAC2-MO1* and *sbHDAC2-MO2*) or the standard control morpholino (10  $\mu$ M) and Endo-Porter (9  $\mu$ M) for 96 h.

**Immunofluorescence Staining of mK4 cells**—Immunofluorescence staining of mK4 cells was performed as described previously (51). The primary antibodies used include an anti-HDAC1 antibody (rabbit polyclonal, 1:100, BioVision) and an anti-HDAC2 antibody (rabbit polyclonal, 1:100, BioVision). For immunofluorescence, the secondary antibody used was Alexa Fluor 555 anti-rabbit (1:1,000, Invitrogen). DAPI (1:500, Invitrogen) was used as nuclear counterstain.

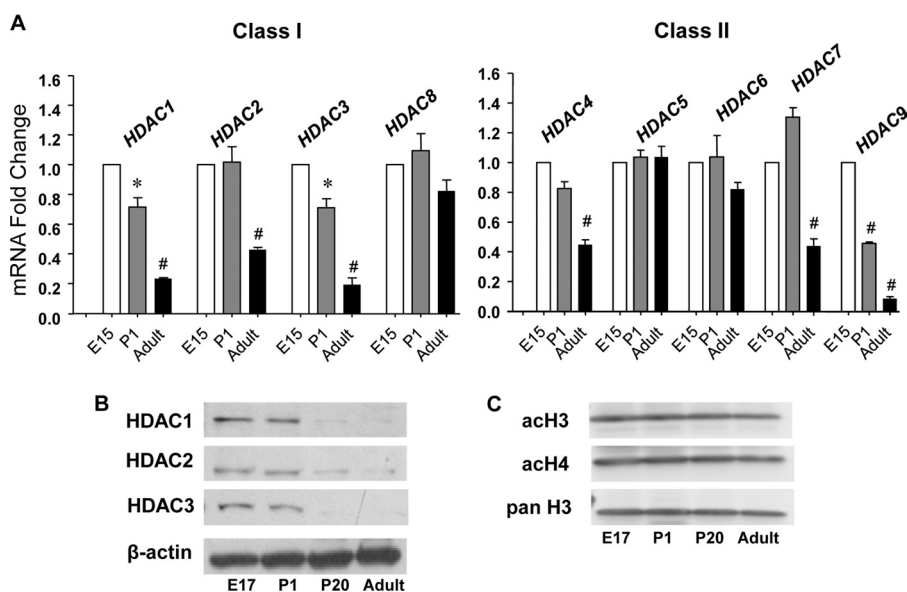
**TABLE 2**  
Primers for ChIP-PCR analysis

Promoter region	Primer sequences
P21 (-514 to -246)	Sense, 5'-TCAAACGACCTTGAATGCCTA-3' Antisense, 5'-GTACAGTTAGAGCTGAGTGAGT-3'
Sfrp1 (-410 to -248)	Sense, 5'-CGAAGGGCTGCTGGTCTGTT-3' Antisense, 5'-TGGGTGGAGTCGGAGTTGGA-3'
Gdnf (-415 to -56)	Sense, 5'-GGTTTATGCCCTACCTTCCC-3' Antisense, 5'-GTGCCCTCCAGTGGCTTTT-3'
Gdnf (-961 to -740)	Sense, 5'-TTCCCTGCCACAATAACT-3' Antisense, 5'-CGGAGAAGGTGAAAGCATAAA-3'
Pax8 (-550 to -328)	Sense, 5'-AGCCAACCGCTTTCCTTA-3' Antisense, 5'-CTCTATGACAACCTTCAACCCCT-3'
Pax2 (-241 to -2)	Sense, 5'-ATCCCTCACCCCTCCCTTTC-3' Antisense, 5'-TGTGTCTCTCTAAAAGCTGC-3'
Pax2 (-492 to -215)	Sense, 5'-TTAGACAGGGGCTGCGGACT-3' Antisense, 5'-GGGTGAAGGAAGAGGGAGGGT-3'
Pax2 (-1692 to -1287)	Sense, 5'-GTTTCTACTTCCAGCAG-3' Antisense, 5'-TTCGCTCCCTCCTCCAGGC-3'
Pax2 (-86245 to -85931)	Sense, 5'-TCCCATGTTGTCGAGGCATG-3' Antisense, 5'-TTCGCTCCCTCCTCCAGGC-3'

**Chromatin Immunoprecipitation (ChIP)**—ChIP experiments were performed by using the EZ ChIP™ chromatin immunoprecipitation kit (Millipore, catalog no. 17-371) according to the manufacturer's protocol. Immunoprecipitation was performed with ChIP grade antibodies to HDAC1 (Abcam, ab7028), HDAC2 (Abcam ab7029), acH3 (Millipore, 06-599), acH4 (Millipore, 06-866), and acH3K9 (Abcam, ab4441). Rabbit IgG (Abcam, ab46540) and mouse IgG (Abcam, ab18413) were used as control antibodies. The chromatin-antibody complexes were captured on protein G-coupled Dynabeads (Invitrogen). After washing and elution of the complexes from the beads, the DNA-protein cross-links were reversed at 65 °C overnight. Next, the precipitated DNA was treated with RNase A and proteinase K and purified using spin columns. The purified DNA along with input genomic DNA (1:100 dilution) were analyzed by PCR. The primer sequences are shown in Table 2.

## RESULTS

**Developmental Expression of HDAC Genes**—We analyzed the expression profiles of class I and II HDAC genes in the mouse kidney by RT-PCR. Among the nine HDAC genes examined, *HDAC1-4*, *-7*, and *-9* are subject to developmental control and decline significantly during the maturation from embryonic to neonatal and adult life (Fig. 1A). We confirmed using Western blot analysis of kidney nuclear lysates that HDAC1–3 proteins are highly abundant in the embryonic kidney and are down-regulated postnatally (Fig. 1B). Interestingly, the global acetylation levels of histones H3 and H4 remain relatively stable during kidney development (Fig. 1C). We also performed RT-PCR screen of HDAC genes in extrarenal organs. For example, *HDAC1-4*, *-7*, and *-9* genes are expressed at higher levels in developing than adult lung (similar to the kidney). However, cardiac and hepatic HDAC genes exhibited only minor developmental regulation (data not shown). Collec-



**FIGURE 1. Developmental expression of the HDAC gene family in mouse kidneys.** A, RT-PCR analysis of class I and II HDAC genes at different ages (E15, postnatal day 1 (P1), and P90). Gene expression levels were normalized to *Gapdh*. The expression level of individual HDAC genes at E15 is arbitrarily set to 1.0 (\*,  $p < 0.05$ , #,  $p < 0.01$  versus E15). B, Western blot analysis of HDAC1–3 proteins in kidney nuclear fractions. C, Western blot analysis of acetylation levels of histones H3 and H4 relative to total histones during kidney development.

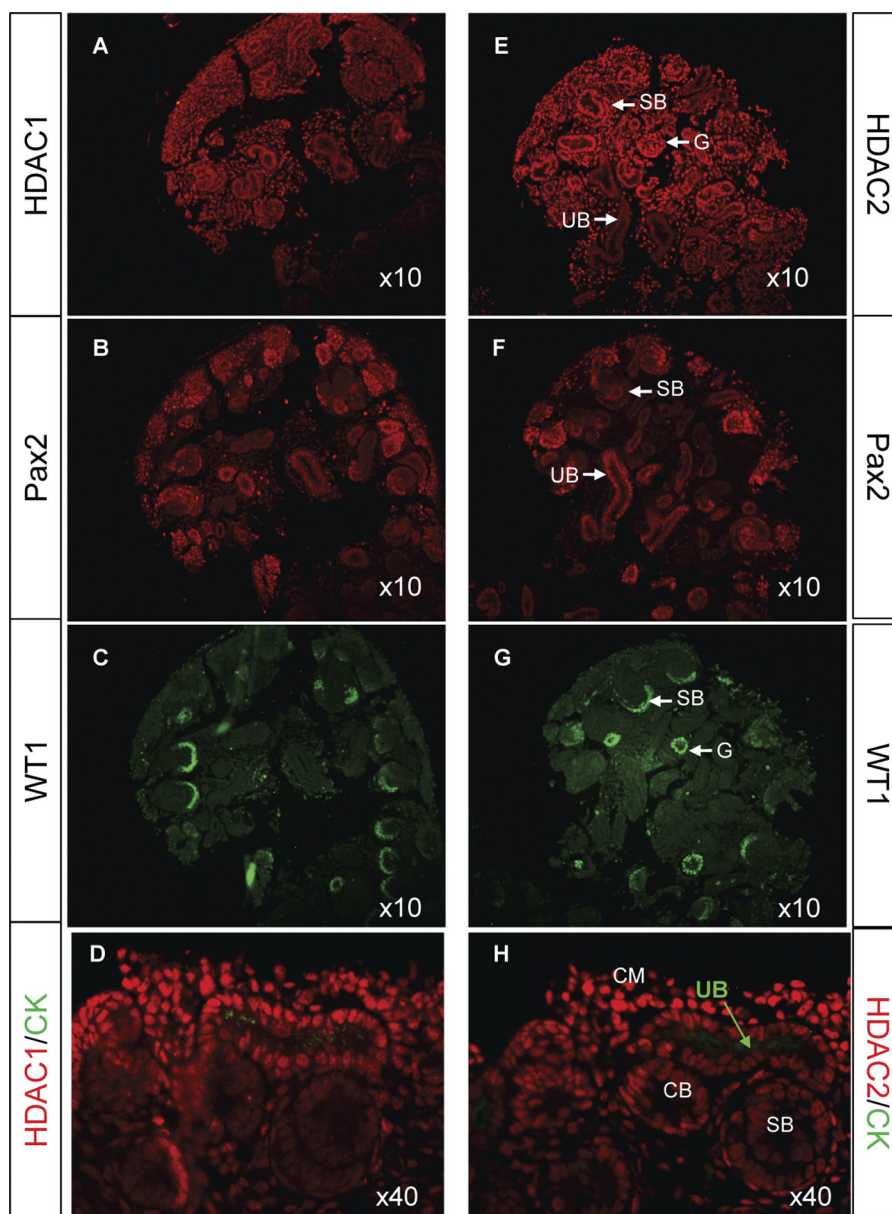


FIGURE 2. **Immunofluorescence localization of HDAC1 and HDAC2 in embryonic kidneys.** A–G, consecutive sections from E13.5 kidneys labeled for HDAC1 or HDAC2, Pax2, and WT1. D and H, E15.5 kidney sections co-labeled with HDAC1 or HDAC2 (red) and cytokeratin (CK), which labels the ureteric bud branches (green). UB, ureteric bud branch; CM, condensing mesenchyme; CB, Comma-shaped body; SB, S-shaped body; G, glomerulus.

tively, our data indicate that class I and class II HDAC genes are differentially regulated during organogenesis. Moreover, maintenance of global histone acetylation levels supports the notion that the balance between histone acetyltransferase/deacetylase activities is tightly regulated during development (52).

**Spatial Expression of HDACs in the Developing Kidney**—To gain insights into potential functions of HDACs, we analyzed their immunolocalization during mouse nephrogenesis. HDAC1 and HDAC2 co-exist in the same protein complex and are believed to perform redundant roles in gene expression (43). Consecutive sections were immunostained for HDAC1, HDAC2, and the renal developmental regulators Pax2 and WT1. On E13.5, HDAC1 and HDAC2 proteins are widely expressed in the undifferentiated MM, nephron progenitors (comma- and S-shaped bodies), interstitial stroma, and UB branches (Fig. 2, A–G). Fig. 2, D and H, depicts a representative

example of E15.5 kidney section co-stained with HDAC1 or HDAC2 and cytokeratin, which labels UB-derived structures. HDAC1 and HDAC2 are expressed in UB branches, condensing mesenchyme, and in comma- and S-shaped nephron progenitors.

In mice, the kidney continues to form new nephrons at the outermost region for few days postnatally, although the deeper region undergoes terminal differentiation. Consecutive sections of PN1 newborn kidneys were stained for HDAC1, HDAC2, HDAC3, Pax2, or WT1. HDAC1 and HDAC2 are expressed in a partially overlapping pattern with Pax2 (Fig. 3, A–F). Moreover, HDAC3 is abundant in the glomerular podocytes (Fig. 3, G–I). This expression pattern is consistent with findings reported by Sharma *et al.* (53). Terminal nephron differentiation, marked by expression of AQP2 and Na,K-ATPase, is associated with reduced expression of HDAC1 and HDAC2

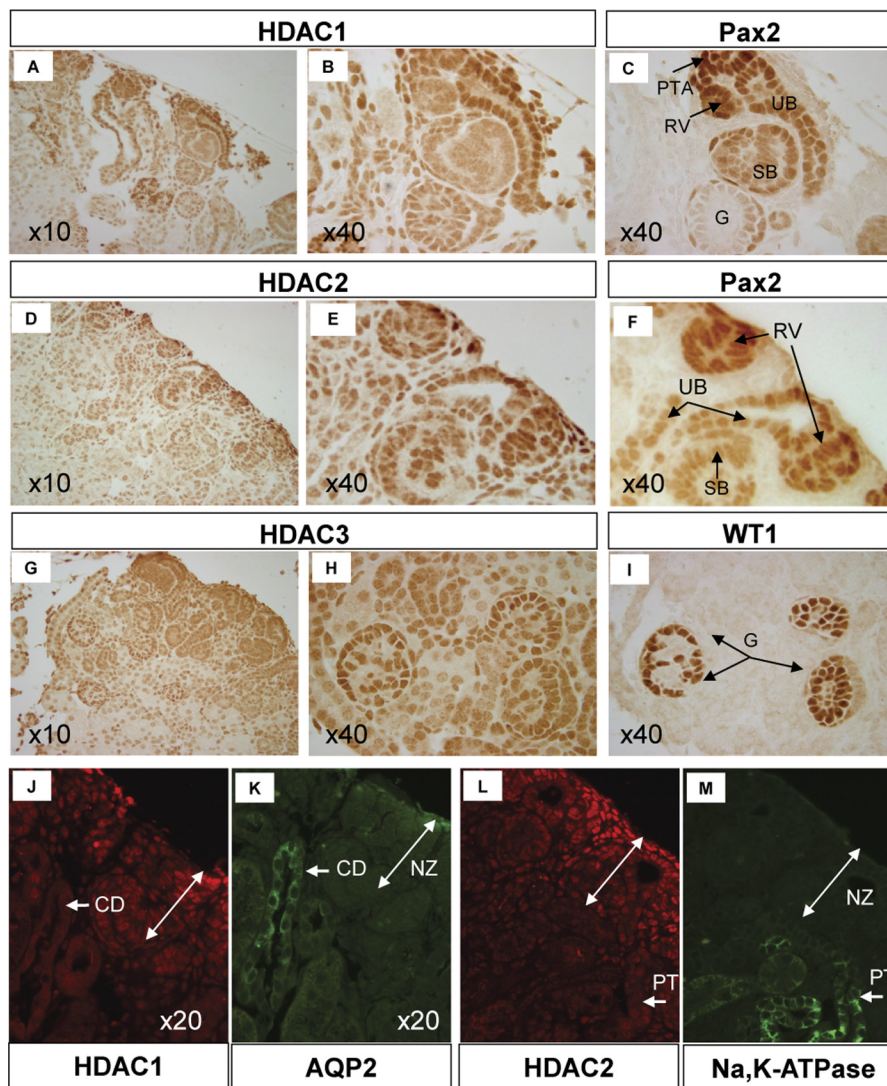


FIGURE 3. **Immunolocalization of HDAC1, HDAC2, and HDAC3 in newborn kidneys.** A–F, consecutive sections stained with HDAC1 or HDAC2 and Pax2 reveal overlapping expression patterns in nephrogenic zone (3,3'-diaminobenzidine immunoperoxidase method). G–I, consecutive sections stained with HDAC3 and WT1 reveal overlapping expression in glomerular podocytes (DAB immunoperoxidase method). J–M, immunofluorescent labeling on consecutive sections for HDAC1 and AQP2 or HDAC2 and Na,K-ATPase, showing enrichment of HDAC1 and HDAC2 in less differentiated nephron structures. PTA, pretubular aggregate; RV, renal vesicle; SB, S-shaped body; G, glomerulus; NZ, nephrogenic zone; CD, collecting duct; PT, proximal tubule.

(Fig. 3, J–M). Similar to other tissues (47, 54), HDAC7 and HDAC8 are predominantly expressed in the renal microvasculature, as indicated by their co-localization with smooth muscle  $\alpha$ -actin (supplemental Fig. 1). HDAC9 is expressed in the peritubular microvascular network (data not shown).

*HDAC Inhibition Stimulates Global Chromatin Histone Acetylation but Causes Selective Effects on Gene Expression in Embryonic Kidneys*—Paired E13.5 mouse kidneys were micro-dissected, cultured *ex vivo*, and treated with the HDACi Scriptaid or its inactive analog Nullscript for 3, 6, or 24 h. Scriptaid, but not Nullscript, induces a time-dependent hyperacetylation of histone H3 and H4 tails (Fig. 4A). To elucidate the pathways affected by HDACi, we carried out a genome-wide microarray analysis on RNA samples extracted from E13.5 embryonic kidneys cultured in the presence of Scriptaid or Nullscript for 6 h. This time point was chosen because it preceded the onset of changes in cell proliferation or survival (see below). The raw and analyzed data have been deposited in the

NCBI Gene Expression Omnibus (GEO), accession number GSE19581. The results revealed that 4,800/41,000 transcripts (~12%) are significantly altered in response to HDACi by  $\geq 1.5$ -fold ( $p < 0.05$ ,  $n = 3$  independent experiments) as follows: 1) up-regulated transcripts ( $n = 2085$ , 5%; range 1.5–14-fold); and 2) down-regulated transcripts ( $n = 2856$ , 7%; range 1.5–26-fold) (Fig. 4B). Ingenuity functional annotation revealed that the deregulated genes were broadly distributed in five major pathways: cell cycle, Wnt/ $\beta$ -catenin, TGF $\beta$ /Smad, cancer, and PI3K/AKT (Fig. 4C). For example, several Wnt ligands and downstream modulators of  $\beta$ -catenin protein (e.g. *Akt* and *p53*) or transcriptional activity (*TCF/Lef* and *CBP/p300*) and Wnt targets (*Myc*, *Cncd1*, and *Axin2*) were down-regulated by HDACi, although the exact intrarenal compartments where these gene expression changes occurred are presently unknown. As expected, HDACi up-regulates the cyclin-dependent kinase inhibitors, *p21* and *p16*, which may account at least partly for their growth-suppressive effect in renal development

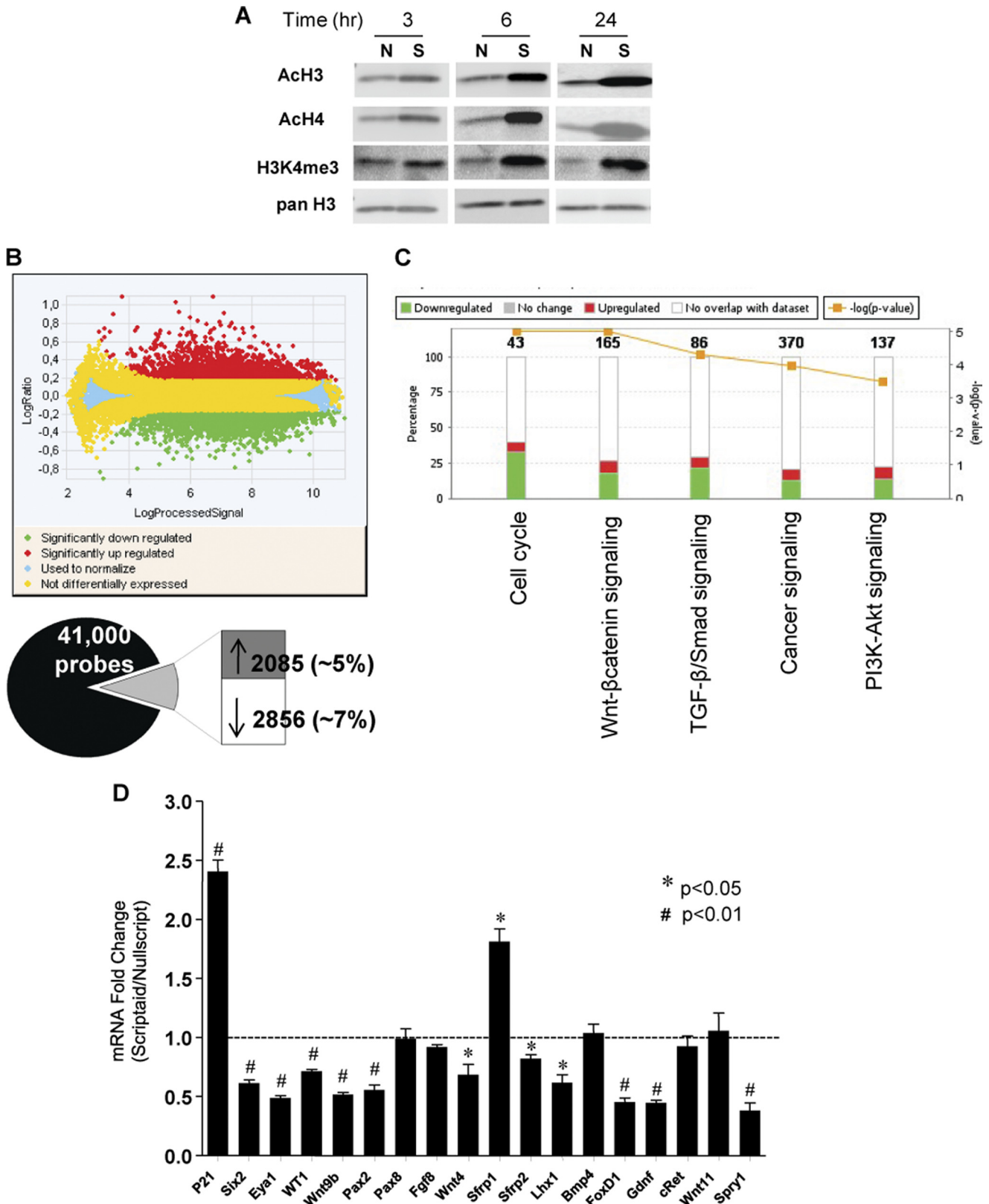


FIGURE 4. **Microarray analysis of differentially expressed genes in response to HDAC inhibition in metanephric kidneys.** *A*, paired E13.5 metanephros were cultured in the presence of Scriptaid (*S*) or inactive analog Nullscript (*N*) 3, 6, or 24 h followed by Western blotting. HDAC inhibition stimulates a time-dependent increase in histone H3 and H4 acetylation as well as H3 trimethylation. *B*, microarray analysis on RNA extracted from Scriptaid- or Nullscript-treated E13.5 kidneys (6 h). HDACi deregulates a subset of genes (12% of 41,000 probes) ( $>1.5$ -fold;  $p < 0.05$ ,  $n = 3$  experiments in triplicates). *C*, ingenuity gene ontology analysis depicts the major biochemical pathways affected by HDAC inhibition. *D*, RT-PCR confirmation of key developmental regulators in Scriptaid- and Nullscript-treated kidney explants.

**TABLE 3**

Selected functional categories of genes decreased in embryonic kidney following HDACi treatment

GO-ID	p value	Description	Genes in test set
48754	2.21E-07	Branching morphogenesis of a tube	NRP1 PGF HOXA11 NPNT GLI2 HOXD11 EDNRA SPRY2 TCF21 NMYC1 CXCR4 BCL2 BMP2 TBX3 TGFB2 FOXA1 GALT IGF1 MMP14 EYA1 TIMELESS NPN1 NOTCH4 PBX1 2310056B04RIK
1822	5.81E-05	Kidney development	IRX3 BMP2 TCFAP2B PGF PKHD1 NPNT HOXA11 GLI2 WT1 HOXD11 TCF21 ACVR2B EYA1 OSR1 LHX1 ITGA8 GATA3 BCL2 PBX1 ROBO2 ADAMTS1 2310056B04RIK FOXO1 LRP4
1658	3.27E-03	Ureteric bud branching	TCF21 EYA1 BMP2 PGF BCL2 NPNT HOXA11 PBX1 2310056B04RIK HOXD11
9880	3.44E-02	Embryonic pattern specification	EDNRA PEG12 NRP1 MEOX2 CXCR4 MEOX1 NPN1 NOTCH4 TGFB2 DLL1 NCKAP1 SIM2
14031	3.51E-02	Mesenchymal cell development	EDNRA HNRPA EDNRB SMO EDN3 BMP2 9130203F04RIK HIF1A ZFXH1B BCL2 SEMA3C

**TABLE 4**

Selected mRNA transcripts significantly altered in embryonic kidney following HDACi treatment

Probe set ID	Gene names	Log fold change	p value
A_52_P177699	<i>Hnf1b</i>	-0.23	0.0024
A_52_P111385	<i>Pbx1</i>	-0.6	0.0011
A_52_P63728	<i>Osr1</i>	-0.25	0.0007
A_52_P36315	<i>Eya1</i>	-0.49	0.0001
A_52_P673458	<i>WT1</i>	-0.29	0.0008
A_51_P102911	<i>Hoxa11</i>	-0.33	0.0025
A_51_P112319	<i>Hoxd11</i>	-0.24	0.0004
A_51_P237783	<i>FoxD1</i>	-0.18	0.0041
A_51_P130475	<i>Wnt4</i>	-0.27	0.0006
A_52_P652336	<i>Lhx1</i>	-0.22	0.0028
A_51_P520849	<i>Sfrp2</i>	-0.3	0.0006
A_51_P147777	<i>Gdnf</i>	-0.16	0.0038
A_52_P546363	<i>Gata3</i>	-0.23	0.0008
A_52_P455251	<i>Emx2</i>	-0.29	0.0003
A_51_P185229	<i>Bmp2</i>	-0.24	0.0004
A_51_P437978	<i>Agtr2</i>	-0.28	0.0042
A_52_P645862	<i>Agtr1a</i>	+0.19	0.0079
A_51_P352296	<i>Sfrp1</i>	+0.32	0.0001
A_52_P220176	<i>Dkk3</i>	+0.23	0.0016
A_51_P484526	<i>Wif1</i>	+0.18	0.0023
A_51_P225224	<i>Htra1</i>	+0.91	0.0001
A_51_P368894	<i>Onecut1</i>	+0.34	0.0002
A_51_P345274	<i>Onecut2</i>	+0.66	0.0002
A_51_P341540	<i>Dnmt3b</i>	+0.33	0.0001
A_51_P449133	<i>Ahr</i>	+0.3	0.0002
A_52_P482897	<i>Areg</i>	+0.36	0.009
A_51_P261991	<i>Bdnf</i>	+0.21	0.0003
A_51_P196925	<i>Cx3cl1</i>	+0.37	0.001
A_52_P573727	<i>Rrm2b</i>	+0.19	0.0005

(see below). Notably, only few pro-apoptotic genes were induced at this time point. Further analysis using the BINGO Network Gene Ontology Tool revealed that many genes involved in kidney development and branching morphogenesis (e.g. *Hoxa11/Hoxd11*, *HNF1b*, *Osr1*, *Gli2*, *Pkhd1*, *Lhx1*, *Gata3*, *Robo2*, *Foxd1*, *Emx2*, *Bmp2*, *Agtr2*, *Spry1*, *TCF21*, *IGF1*, *CXCR4*, *MMP14*, *Eya1*, *Timeless*, *Notch4*, and *Pbx1*) were down-regulated following HDACi treatment (Tables 3 and 4). Other developmental regulators, such as *Htra1*, *Dkk3*, and *Sfrp1*, were up-regulated in response to HDACi treatment. Similarly, a number of differentiation factors such as metabolic enzymes and G-protein-coupled receptors, were up-regulated (see examples in Table 4). Semi-quantitative RT-PCR performed on 18 developmental renal regulators confirmed the microarray results (Fig. 4D).

*Expression of Renal Developmental Regulators Is Critically Dependent on HDAC Activity*—We conducted a time course study using whole mount ISH to assess the spatiotemporal effects of HDACi on key gene regulatory networks. The *Eya1* gene encodes a transcription co-factor and is one of the earliest markers of the MM (55, 56). *Eya1* interacts with *Pax2* and *Six1* in regulation of *Six2* and *Gdnf* (7, 57). *Six2* is required for the self-renewal of the cap MM, which gives rise to nephron progenitors, whereas the *Gdnf*-Ret axis is required for UB branch-

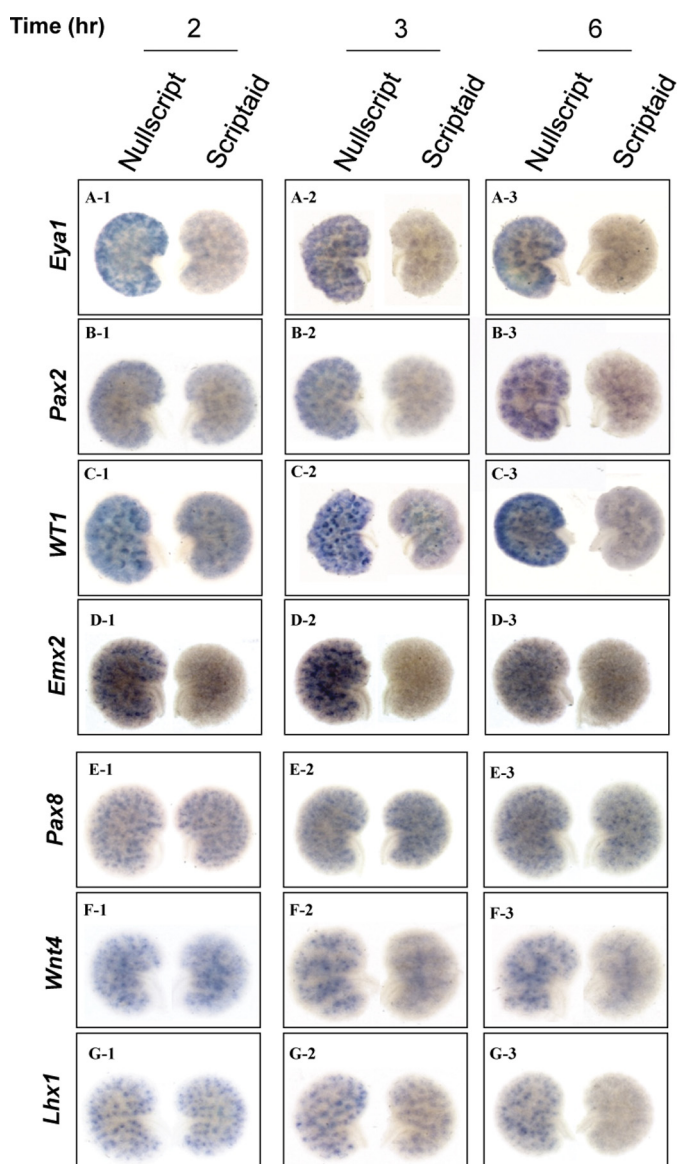
ing morphogenesis (25, 26). Genes encoding the transcription factors, *WT1*, *Emx2*, and the *Hox11* paralogs are crucial for MM induction and survival, and their inactivation results in renal agenesis (58–60). Fig. 5 demonstrates that *Eya1* (*A-1* to *A-3*), *Pax2* (*B-1* to *B-3*), *WT1* (*C-1* to *C-3*), and *Emx2* (*D-1* to *D-3*) are among the most sensitive genes to HDACi, because their expression is down-regulated as early as 2 h following HDACi treatment.

The canonical Wnt-β-catenin signaling pathway plays a key role in patterning kidney development during mesenchymal-epithelial transition (23, 24, 36). Wnt9b, released from UB branches, mediates the conversion of MM cells into epithelial vesicles that express *Pax8*, *Wnt4*, and *Fgf8* (23). The latter factors, in conjunction with Notch signaling and *Lhx1* (also known as *Lim-1*), mediate further nephron maturation and segmental identity (29, 61). The results shown in Fig. 5 demonstrate that *Wnt4* (*F-1* to *F-3*) and *Lhx1* (*G-1* to *G-3*) expression is down-regulated 3 h post-HDACi treatment. With regards to *Pax8*, in the organ culture system, *Pax8* was slightly down-regulated 6 h post-HDACi treatment by ISH (Fig. 5E-3), whereas the microarray and RT-PCR failed to detect the decrease of *Pax8* (Fig. 4). Likely, this is a sensitivity issue, and whole mount ISH may be a more sensitive approach to detect subtle focal changes in gene expression in the renal vesicles. It was in the mK4 cells that *Pax8* was dramatically repressed 6 h post-HDACi treatment (Fig. 7A); perhaps there are compensatory factors in the complex organ culture that maintain *Pax8* expression in response to HDACi.

The reasons for the temporal differences in gene expression changes among various developmental regulators are not completely known. Because both compartments express class I HDAC enzymes, we speculate that this differential sensitivity to HDACi may be related to gene-specific differences in accessibility of HDACs to chromatin, differences in proliferative state between early and late progenitors, or possibly to slight differences in drug concentrations/penetration among the various compartments.

In comparison with the exquisite sensitivity of MM and nephron progenitor genes to HDACi, expression of *Foxd1* (a stromal transcription factor required for kidney patterning and UB branching) and *Bmp4* (a growth and differentiation factor required for both stromal and ureter development) is minimally affected by HDACi (Fig. 6, *A-1* to *A-3* and *B-1* to *B-3*). In a similar line, *c-ret*, a *Gdnf* receptor tyrosine kinase, and its downstream target *Wnt11*, which drive UB outgrowth and branching morphogenesis, are relatively resistant to HDACi (at least at this stage of kidney development) (Fig. 6, *C-1* to *C-3* and *D-1* to *D-3*). This is in contrast to two other UB-expressed genes *Spry1* and *Wnt9b*, both expressed in UB tip/branches, which

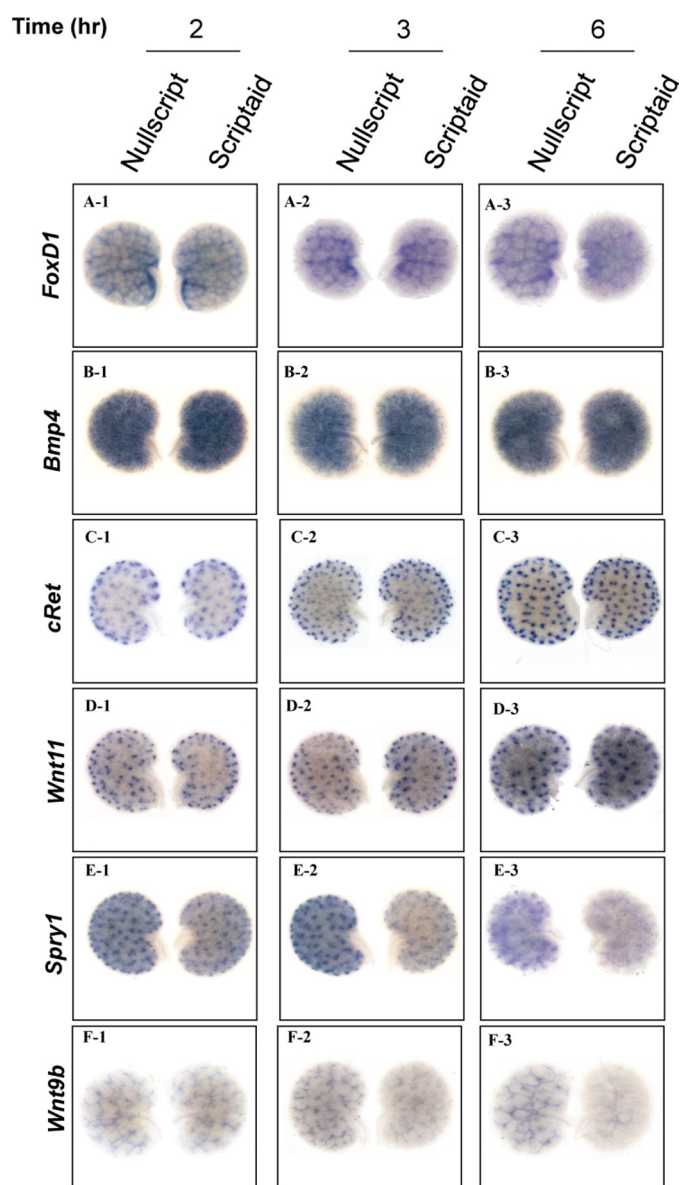




**FIGURE 5. Time course analysis of the effects of HDACi on developmental renal regulators (nephron progenitors).** Paired E13.5 kidneys were cultured in the presence of Scriptaid or Nullscript for the indicated time points and processed for *in situ* hybridization with the indicated gene probes. *Eya1*, *Pax2*, *WT1*, and *Emx2* are highly sensitive to HDAC inhibition (down-regulated at 2 h post-HDACi), followed by *Wnt4* and *Lhx1* (3 h) and *Pax8* (6 h).

are repressed by HDACi (Fig. 6, *E-1* to *E-3* and *F-1* to *F-3*). Taken together, our microarray data, RT-PCR and ISH results, demonstrate that the transcriptome of the developing kidney is selectively regulated by HDACs and that renal developmental regulators exhibit differential sensitivity to HDACi. Moreover, our results are consistent with the notion that many developmental regulators are dependent on HDAC activity for their expression and that HDACi can either up-regulate or repress gene transcription.

**Effect of HDACi on Chromatin Acetylation of Selected Renal Developmental Regulators**—We next asked whether the effects of HDACi on expression of renal developmental regulators can be reproduced in cultured MM cells and, if so, whether these effects are accompanied by promoter-specific changes in histone acetylation, thus linking HDACs with physiological regu-



**FIGURE 6. Time course analysis of the effects of HDAC inhibition on developmental renal regulators (stroma and UB).** Paired E13.5 metanephroi were cultured in the presence of Scriptaid or Nullscript for the indicated time points and processed for *in situ* hybridization with the indicated gene probes. *FoxD1* expression is repressed 6 h post-HDACi, and *Bmp4*, *c-ret*, and *Wnt11* are maintained. In contrast, *Spry1* and *Wnt9b* are sensitive to HDACi and are down-regulated by 2–3 h post-HDACi.

lation of these genes. mK4 cells (immortalized mouse MM cells (62)) were cultured in the presence of Scriptaid (2  $\mu$ g/ml) or control drug, Nullscript (2  $\mu$ g/ml) for 6 h followed by RNA extraction and RT-PCR. The results were expressed as relative mRNA levels of Scriptaid/Nullscript. Fig. 7A shows that HDACi treatment of mK4 cells for 6 h activates or represses similar sets of genes as compared with HDACi-treated embryonic kidneys (compare with Fig. 4D). Western blot analysis of chromatin-extracted proteins using antibodies to H3, aceH3, and aceH4 revealed the expected global histone lysine hyperacetylation (Fig. 7B). As reported previously, this is accompanied by stimulation of H3K4 trimethylation (Fig. 7B), which is mediated in part from transcriptional down-regulation of H3K4 demethylases (63).

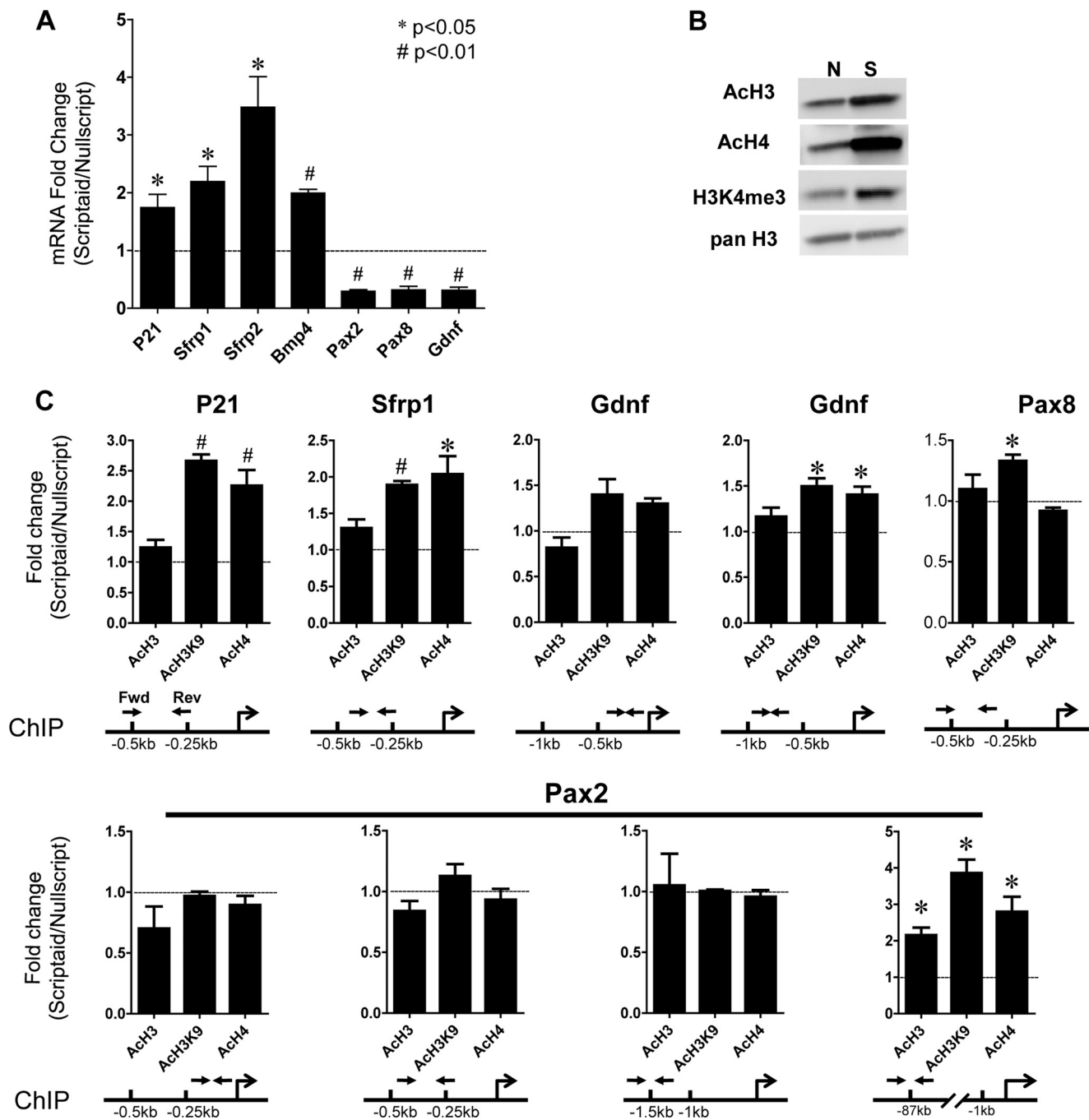


FIGURE 7. **Direct actions of HDAC inhibition on mK4 gene expression.** *A*, analysis of gene expression. Metanephric mesenchyme mK4 cells were cultured in the presence of Scriptaid or control drug Nullscript for 6 h followed by RNA extraction and RT-PCR. The majority of the gene expression changes is similar to those observed metanephric organ cultures treated with HDACi (compare with Fig. 4D). *B*, treatment of mK4 cells with Scriptaid (S), but not Nullscript (N) for 6 h stimulates H3 and H4 hyperacetylation and H3K4 trimethylation. *C*, ChIP assays. Chromatin immunoprecipitation was performed in mK4 cells using antibodies to acetyl-H3, acetyl-H3K9, and acetyl-H4 following treatment with Scriptaid or Nullscript. PCR density was factored to input, and results were expressed as fold change in relative enrichment of histone marks in Scriptaid/Nullscript-treated samples.

We next examined the effect of HDACi on acetylation of promoter histones of selected target genes (*p21*, *Sfrp1*, *Gdnf*, and *Pax2*, and *Pax8*). ChIP analysis was performed on mK4 cells treated with Scriptaid or Nullscript for 6 h utilizing ChIP-grade antibodies to AcH3, AcH3K9, AcH4, HDAC1, and HDAC2. Controls consisted of ChIP performed with rabbit or mouse IgG. Input DNA was used to correct for the amount of recovered DNA. Semi-quantitative PCR was performed on five selected genes (two up-regulated and three down-regulated)

using gene-specific primers (Table 2). The experiments were performed at least three times, and the densitometric intensity of the PCR products was averaged and compared between Scriptaid- and Nullscript-treated samples after factoring for input and IgG controls and using a *p* value of <0.05 as a cutoff for statistical significance. Fig. 7C and supplemental Figs. 2 and 3 show that promoter/enhancer hyperacetylation was significantly stimulated in all five genes. Thus, HDACi likely exerted a direct effect on promoter histone acetylation. In support of this

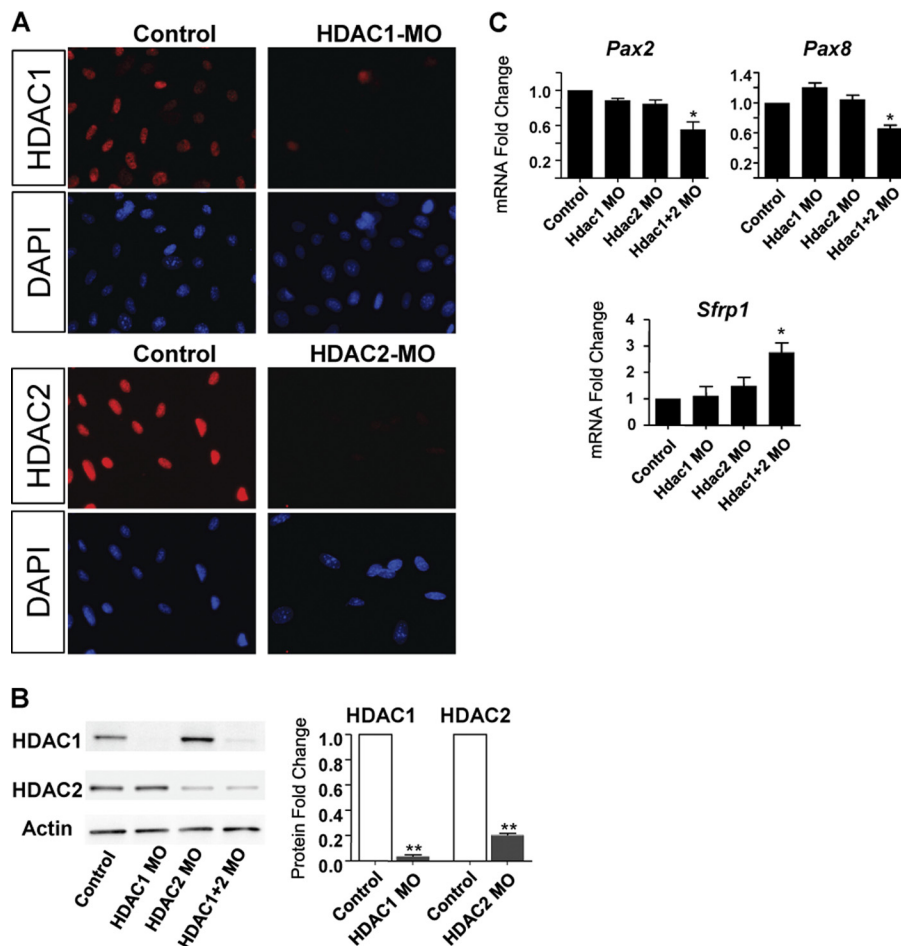


FIGURE 8. Effects of MO-mediated gene silencing of HDAC1 and HDAC2 on gene expression in mK4 cells. A, immunofluorescence staining of HDAC1 and HDAC2 proteins in mK4 cells transfected with HDAC1/HDAC2 antisense MO or control oligonucleotides. B, quantitative analysis of HDAC1 and HDAC2 protein levels by Western blotting. mK4 cells were treated with HDAC1-MO (10  $\mu$ M) and/or HDAC2-MO (10  $\mu$ M each of HDAC2-MO1 and HDAC2-MO2) for 96 h. Standard control morpholinos (10  $\mu$ M) were used as negative control. \*\*,  $p < 0.01$ . C, real time RT-PCR analysis of gene expression. mK4 cells were treated with morpholinos as described in B. RNA was extracted, and relative gene expression of *Pax2*, *Pax8*, and *Sfrp1* was quantified by quantitative RT-PCR ( $n = 3$  independent experiments in triplicate). \*,  $p < 0.05$ .

conclusion, the promoters of all five genes are occupied by HDAC1 and HDAC2, although HDACi did not appear to have a significant effect on their relative binding to chromatin (supplemental Figs. 2 and 3).

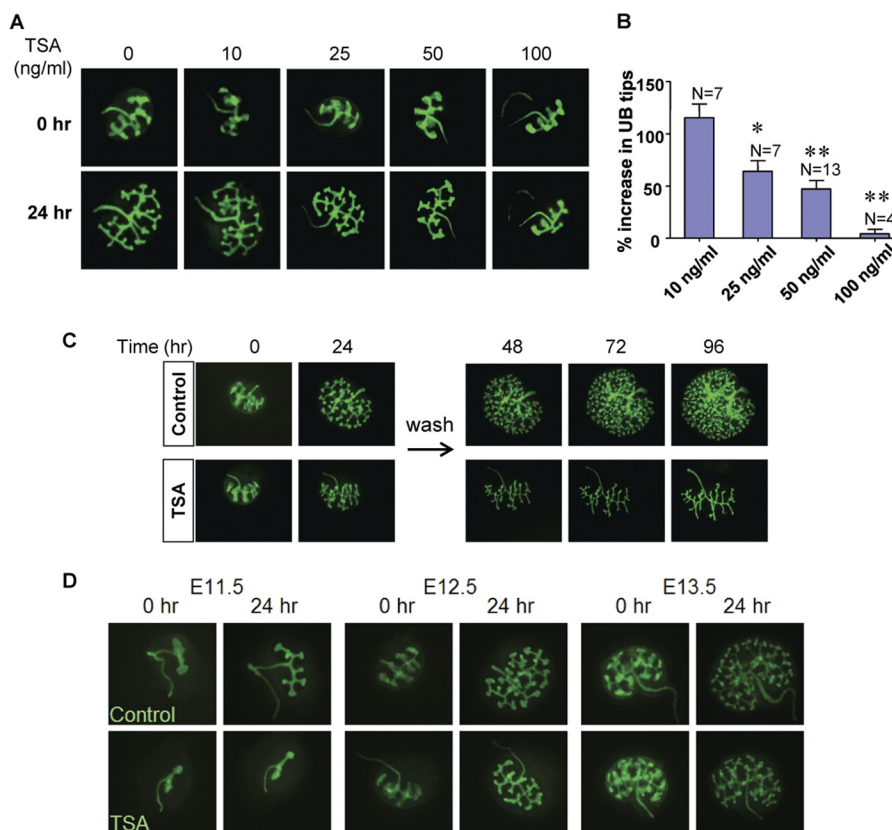
**HDAC1 and HDAC2 Regulate the Developmental Renal Regulators *Pax2*, *Pax8*, and *Sfrp1***—The transcription factors *Pax2* and *Pax8* play key roles in renal development (3, 64). *Pax2* is abundantly expressed in nephron precursors and overlaps spatially with HDAC1 and HDAC2 (e.g. Figs. 2 and 3). Increasing evidence indicates that HDAC1 and HDAC2 have distinct as well as redundant functions in development and disease (43). Here, we examined the individual as well as combined loss of function of HDAC1 and HDAC2 using antisense MO. MOs are complementary to the translation start site and thus block protein synthesis. Fig. 8, A and B, shows that MO-mediated knockdown is highly efficient and specific in silencing HDAC1 and HDAC2, as assessed by immunofluorescence and Western blotting. Although knockdown of either HDAC1 or HDAC2 is not associated with discernible effects, double HDAC1/HDAC2 knockdown significantly represses *Pax2* and *Pax8* and up-regulates *Sfrp1* gene expression in mK4 cells ( $n = 3$ ;  $p < 0.05$ ) (Fig. 8C), thus mimicking the observed effects of HDACi

on expression of these developmental regulators (Fig. 7A) and providing further direct evidence for the role of class I HDACs in the regulation of gene expression in the developing nephron.

**HDAC Inhibition Disrupts Renal Development**—The metanephric organ culture recapitulates nephrogenesis and UB branching *in vivo* (65). To avoid the effects of HDACi on the fetoplacental unit, we treated freshly harvested and cultured embryonic kidneys with HDACi and followed their growth and development over time. We utilized three well characterized HDACi drugs; TSA and Scriptaid inhibit both class I and II HDACs, whereas MS-275 is a selective inhibitor of HDAC1–3. To control for the nonspecific effects of Scriptaid, we compared Scriptaid to its inactive structural analog Nullscript. Given the variability in kidney size among littermates, we compared the effects of HDACi on paired kidneys from each embryo. Moreover, to enable real time monitoring of UB branching morphogenesis by fluorescence microscopy, we used *Hoxb7*-GFP<sup>+</sup> transgenic mice, which express GFP exclusively in the UB lineage (66).

Paired kidneys were dissected from *Hoxb7*-GFP<sup>+</sup> mouse embryos at *E11.5*–*E13.5* and cultured in the presence or absence of various concentrations of HDACi. Assessment of

## Histone Deacetylases in Renal Development



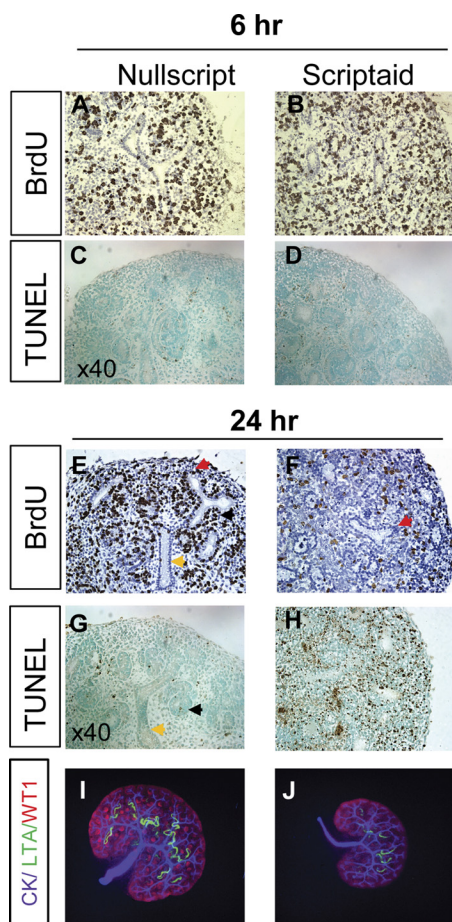
**FIGURE 9. Effect of the HDAC inhibitor, TSA, on UB branching in metanephric organ culture.** *A*, Hoxb7-GFP<sup>+</sup> metanephroi were microdissected on E12.5 and cultured *ex vivo*. The kidneys were treated with TSA or vehicle (DMSO) at various concentrations (10, 25, 50, and 100 ng/ml) for 24 h. *B*, bar graph shows the effect of TSA on the percent increase in the number of UB tips over the ensuing 24 h (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). *C*, representative sequential images of UB branching in control (DMSO) and TSA-treated Hoxb7-GFP<sup>+</sup> metanephroi over 96 h in culture. TSA was washed off at 24 h. *D*, effects of TSA on UB branching at various stages of renal development. Hoxb7-GFP<sup>+</sup> metanephroi were microdissected at E11.5, E12.5, and E13.5, and cultured *ex vivo* in the presence of TSA or DMSO (control).

UB branching was performed by counting the absolute number of UB branches/tips and calculating the percent increase in tip number during the ensuing 24–96 h in HDACi *versus* control explants. TSA-treated kidney rudiments exhibited stunted UB branching in a dose-dependent manner (Fig. 9, *A* and *B*). Similar results were obtained using Scriptaid and the class I HDACi MS-275 (supplemental Figs. 5 and 6).

We next asked whether the effects of HDACi on renal growth are reversible. Paired E12.5 Hoxb7-GFP<sup>+</sup> kidneys were cultured in the presence of TSA (50 ng/ml) or vehicle for 24 h followed by extensive washing and culture for an additional 72 h. Fig. 9*C* depicts that unlike vehicle-treated kidneys, which branched extensively, minimal branching occurred in TSA-treated kidneys at 24 h, and it was not reversible upon removal of TSA by extensive washing. Additional time course studies revealed that the effects of HDACi on metanephric growth become irreversible after ~12 h of exposure to HDACi (data not shown). Thus, there is a window of time during which the effects of HDACi on nephron growth remain reversible. Of note, the effects of HDACi on overall UB branching seem to be more profound when applied during earlier stages of kidney development. For example, treatment of E11.5 kidney (T-stage branching) with HDACi completely arrests further branching, whereas treatment at later developmental stages produces less profound effects on UB branching (Fig. 9*D*).

Because *Gdnf* gene expression is down-regulated in HDACi-treated kidneys, we asked whether exogenous replacement of high dose *Gdnf* (200 ng/ml) can rescue kidney growth. This dose of *Gdnf* is capable of inducing robust ectopic UB outgrowth and branching (67). However, E13.5 metanephric explants treated with Scriptaid + high dose *Gdnf* (200 ng/ml) for 24 h failed to show any rescue of growth defect (supplemental Fig. 6). Thus, *Gdnf* deficiency alone does not account for HDACi-induced growth impairment and is consistent with the microarray results showing significant alterations in multiple developmental pathways.

**Effects of HDAC Inhibition on Metanephric Cell Proliferation and Apoptosis**—We examined cell proliferation and apoptosis in paired E13.5 kidneys following 6 and 24 h of treatment with Scriptaid or Nullscript. The rates of BrdU incorporation and TUNEL staining were used as measures of cell proliferation and apoptosis, respectively. In Nullscript-treated E13.5 metanephroi, BrdU incorporation is observed throughout the organ (Fig. 10, *A* and *E*). In contrast, only occasional cells were TUNEL-positive (Fig. 10, *C* and *G*). Neither cell proliferation rate nor cell death was affected following 6 h of Scriptaid treatment (Fig. 10, *B* and *D*). Conversely, at 24 h, cell proliferation was greatly diminished and was accompanied by a remarkable increase in the number of TUNEL-positive cells (Fig. 10, *F* and *H*). Immunostaining for markers of proximal tubule (*L. tet-*



**FIGURE 10. Effect of HDAC inhibition on metanephric cell proliferation and apoptosis.** Paired E13.5 kidneys were cultured in the presence of Scriptaid or control drug Nullscript (2  $\mu\text{g}/\text{ml}$ ) for 6 or 24 h. Metanephroi were processed for *in situ* TUNEL assays or pulsed for the last 2 h of culture with BrdU. *A–D*, HDAC inhibition for 6 h has no discernible effects on metanephric cell proliferation or apoptosis. *E–H*, in contrast, HDAC inhibition for 24 h stunts metanephric growth and is accompanied by widespread cell growth arrest and apoptosis. Red arrowheads, UB branches/tips; yellow arrowhead, UB stalk; black arrowhead, developing nephron. *I* and *J*, E13.5 metanephroi cultured in the presence of Scriptaid or Nullscript (2  $\mu\text{g}/\text{ml}$ ) for 24 h followed by co-labeling of *L. tetragonolobus* lectin agglutinin (*LTA*) (green, marker of proximal tubule), WT1 (red, marker of podocytes/glomeruli), and cytoke-  
 ratin (*CK*, purple, marker of UB branches). Proximal tubule formation, glomerulogenesis, and UB branching are all inhibited by Scriptaid as compared with its inactive structural analog Nullscript.

*ragonolobus* lectin agglutinin), glomeruli (WT1), and UB epithelium (cytoke-  
 ratin) revealed significant growth-stunting and poor proximal tubule and glomerular development in Scriptaid-treated as compared with Nullscript-treated explants (Fig. 10, *I* and *J*).

## DISCUSSION

This study investigated the developmental expression of HDACs and the effects of HDAC inhibition on gene expression and development of the embryonic kidney. Our major findings are as follows. 1) The developing kidney expresses nine members of the class I and II HDAC family of enzymes. 2) Based on their spatiotemporal expression patterns and the effects of HDACi and gene knockdown, class I HDACs are candidate regulators of metanephric development. 3) HDACs regulate essential genes involved in renal growth and differentiation. 4)

Genome-wide analysis revealed HDAC inhibition can either stimulate or repress metanephric gene transcription.

**Developmental Expression of HDACs**—Our results demonstrate that several HDAC genes are developmentally regulated. These include HDAC1–4, -7, and -9. In comparison, HDAC5, -6, and -8 are constitutively expressed. The developmental down-regulation of class I HDAC genes correlates with the known reduction in proliferative activity during nephron differentiation, reminiscent of intestinal villus differentiation. In the mouse intestinal epithelium, HDAC2 and HDAC3 are highest in abundance in the proliferating crypt cells and decline during villus maturation (68). Overexpression of HDAC1 and HDAC2 in cultured intestinal explants delays expression of differentiation markers, whereas HDACi provokes premature differentiation (69). In this regard, our data indicate that high HDAC activity in the embryonic kidney is required for expression of cell proliferation and survival pathway genes, such as *c-myc*, cyclin D1, and thymidylate kinase, and for suppression of tumor suppressors and cell cycle inhibitors, such as *p21*, *p15*, and *p16*.

**Effect of HDACi on the Embryonic Renal Transcriptome**—Genome-wide profiling revealed that 12% of the embryonic kidney transcriptome is HDAC-regulated (using a cutoff of 1.5-fold,  $p < 0.05$ ). Bioinformatic analysis revealed several interesting observations. First, most differentially expressed genes fall under the cell cycle, Wnt/ $\beta$ -catenin, TGF- $\beta$ /Smad, and PI3K-AKT, pathways. Considering the importance of these biological pathways in organ growth and morphogenesis, it is not surprising that exposure of kidney explants to HDACi led to hypoplasia accompanied by suppressed cellular proliferation and enhanced apoptosis. Second, ~60% of the differentially expressed genes in response to HDACi were repressed. It is commonly assumed that HDACs function as repressors of gene transcription. However, there is increasing evidence that HDACs exhibit both repressive and activating effects on gene transcription (37, 70). In this regard, global HDAC inhibition or deletion of individual HDAC genes in mice or embryonic stem cells affects expression of up to 10% of the expressed cellular genes, and nearly as many genes are down-regulated as are up-regulated (40, 71–73). Our findings are consistent with the notion that HDACs can either stimulate or repress gene transcription, and the widely held view that HDACs function predominantly as transcriptional repressors should be reconsidered. Mechanistically, specific patterns of histone lysine acetylation may cooperate with other forms of histone modifications (e.g. methylation) to create a “histone signature” for recruitment of transcriptional regulators (74–76). Our ChIP analysis and gene knockdown studies support the idea that HDACs regulate directly at least a subset of genes involved in nephron differentiation. However, HDACs also act via nongenomic mechanisms as well. For example, HDACs deacetylate transcription factors, such as p53, STAT3, YY1, GATA1, E2F1, and other proteins like tubulin and Hsp90 (77). Thus, the effects of HDACi on gene expression may be direct (*i.e.* epigenetic) or indirect (via nongenomic effect) or both.

The third important finding of our study is that HDAC activity is required for basal expression of key renal developmental regulators, such as *Eya1*, *WT1*, *Pax2*, *Hox11* paralogs, *HNF1 $\beta$* ,

## Histone Deacetylases in Renal Development

*FoxD1*, *Wnt9b*, *Gdnf*, and downstream epithelial differentiation effectors, e.g. *Pax8*, *Fgf8*, *Wnt4*, *Lhx1*. Time course analysis in organ culture revealed that some developmental genes, e.g. *Pax2*, *WT1*, *Eya1*, *Wnt9b*, and *Wnt4*, are more sensitive to HDACi than others, e.g. *c-ret* or *Bmp4*, despite the widespread expression of HDACs in the developing kidney. Although the exact reason is presently unknown, this finding suggests that HDACs may differentially bind to and/or regulate different target genes in the developing nephron.

Our organ culture studies suggest that HDAC activity is required to maintain active canonical Wnt signaling in metanephric kidneys. We speculate that this function is mediated via *Wnt9b*. Our data show that *Wnt9b* is among the earliest genes down-regulated in HDACi-treated metanephroi and may account for repression of  $\beta$ -catenin-target genes, *Axin2*, *c-myc*, and cyclin D1, as well as renal vesicle genes *Fgf8*, *Pax8*, *Wnt4*, and *Lhx1*. In a recent study, De Groh *et al.* (48) reported in zebrafish that HDACi expands the domain of pronephric developmental regulators and renal progenitor cells in a retinoic acid-dependent manner.

**HDACs and Metanephric Cell Proliferation/Apoptosis**—This study demonstrates that sustained inhibition of HDAC activity (>12 h) blocks cell proliferation and induces cell apoptosis in cultured kidney explants. The mechanisms of HDACi-induced growth arrest and apoptosis have been extensively investigated. In cancer cell lines, HDACi-induced cell cycle arrest at G<sub>1</sub>/S and G<sub>2</sub>/M is commonly associated with activation of CDK inhibitors *p21*, *p19*, and *p1*, and repression of cyclin D1, *c-myc*, and thymidylate synthase. Also, HDACi can transcriptionally activate the extrinsic and intrinsic pathways of apoptosis (38, 77).

In this study, we observed that CDK inhibitors/tumor suppressor genes, including *p21*, *p15*, *p19*, *Bop1*, and *Htra1*, were significantly up-regulated by HDACi. Moreover, many oncogenes, such as *c-myc*, *N-myc*, cyclin D1, cyclin J, cyclin B2, and thymidylate synthase, were dramatically down-regulated. Thus, the imbalance between growth promoters and suppressors can collectively contribute to metanephric growth arrest induced by HDACi. The apoptosis-regulating genes, *Bcl-2* and caspase 9, exhibited modest down- and up-regulation, respectively, in response to HDACi. Although we cannot completely rule out nonspecific effects related to the use of pharmacological inhibitors, morpholino-mediated knockdown of HDAC1 and HDAC2 strongly suggests that class I HDACs regulate gene expression in MM cells. Ongoing studies in our laboratory utilizing nephric lineage-specific inactivation of individual HDACs will be paramount in delineating the role of individual HDACs in renal development.

In summary, multiple lines of evidence derived from our study and others support the hypothesis that HDACs play important roles in nephrogenesis. HDAC1–3, which play key roles in regulating cell proliferation and cell survival in other organs such as intestines, liver, and brain, are also enriched in nephron progenitors and UB branches. Utilizing two class I/II HDAC inhibitors and a more selective class I HDAC inhibitor, we show that inhibition of HDAC activity impairs renal growth. Inhibition of HDAC activity alters expression of developmental regulators involved in MM competence and survival, mesen-

chyme-to-epithelium differentiation, and proliferation. Finally, our findings support the notion that HDAC1 and HDAC2 perform important yet redundant functions in regulation of embryonic kidney cell gene expression, as described previously in other cell types.

*Acknowledgments*—We are grateful to O. Wessely (Louisiana State University Health Sciences Center) and members of the El-Dahr laboratory for advice, reagents, and discussions.

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