Histone deacetylase 1 and 2 regulate Wnt and p53 pathways in the ureteric bud epithelium

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INTRODUCTION
Histone deacetylases (HDACs) are a superfamily of enzymes important for modulation of chromatin structure and function via removal of acetyl groups from histones as well as non-histone proteins. In higher eukaryotes, 18 HDAC isoforms have been identified. Based on sequence homology to yeast HDAC genes, they are divided into four classes: the class I RPD3-like HDACs (1-3 and 8); the class II HDA1-like HDACs (4-7, 9 and 10); Sirtuins 1-7; and the class IV HDAC11 (de Ruijter et al., 2003; Denu and Gottesfeld, 2012). HDACs can also deacetylate an increasing number of non-histone proteins, including p53, STAT3, Yin Yang transcription factor (YY1), GATA1, E2F1 and Hsp90 (Glozak et al., 2005; Kruse and Gu, 2009; Spange et al., 2009). Deacetylation of these proteins has been shown to affect multiple aspects of their function, such as protein stability, DNA binding affinity and transcriptional activity, adding an extra layer of complexity to the biological roles of HDACs.

In order to delete the Hdac1 and Hdac2 alleles in the UB cell lineage, we revealed the redundant yet essential cell-autonomous functions of Hdac1 and Hdac2 in the ureteric epithelium during mouse kidney development. Moreover, our findings illustrate the developmental importance of HDAC-mediated control of p53 acetylation.

RESULTS
Concurrent deletion of Hdac1 and Hdac2 in the UB cell lineage causes renal hypodysplasia
In order to delete the Hdac1 and Hdac2 genes specifically from the UB lineage, we crossed $\text{Hdac1}^{\text{flox/flox}}$, $\text{Hdac2}^{\text{flox/flox}}$ and...
**RESEARCH ARTICLE**


**Hoxb7-CreEGFP transgenic mice (Montgomery et al., 2007; Zhao et al., 2004). Previous studies have shown that Hoxb7-directed GFP expression is observed in the Wolffian duct at embryonic day (E) 10.0 and its derivatives, the UB and its branches, but not in the MM lineage (Zhao et al., 2004). To test the efficacy of Hoxb7-driven Cre-mediated excision, we examined the expression of Hdac1 and Hdac2 proteins by immunohistochemistry in wild-type and mutant kidney tissues at E13.5. Consistent with our previous report, Hdac1 and Hdac2 are expressed in both the UB and MM cells in wild-type and mutant kidneys (Fig. 1A,E). By contrast, in UB^Hdac1,2−/− mice (Hoxb7-Cre^tg/+; Hdac1^flox/flox; Hdac2^flox/flox), Hdac1 and Hdac2 are not detected in the UB cells but are maintained in the surrounding mesenchymal cells (Fig. 1B,F). In accordance with the key functions of Hdac1 and Hdac2 in histone deacetylation, the acetylation levels of histone H3 (lysine 9 and 14, or lysine 9 specifically) and H4 (lysine 5, 8, 12 and 16) are substantially increased in the ureteric cells of UB^Hdac1,2−/− kidneys (Fig. 1I-N). Collectively, these results demonstrate efficient deletion of Hdac1 and Hdac2 from the UB branches. It is worth noting that knockout of Hdac1 and Hdac2 in oocytes leads to no apparent change in histone H3K9 acetylation (Ma et al., 2012). Thus, our results suggest that Hdac1 and Hdac2 might have different histone residue specificity in different cell types at specific developmental stages.

Our results revealed that mice with no more than three deleted alleles of Hdac1 and Hdac2 exhibit no significant abnormalities in kidney development (supplementary material Fig. S1); moreover, these mice survive to adulthood without any overt abnormalities in growth or development. By contrast, concurrent deletion of all four alleles of Hdac1 and Hdac2 results in early postnatal lethality by 2-4 weeks of age (supplementary material Fig. S2). Histological analysis of kidney tissue from UB^Hdac1,2−/− mice at postnatal day (P) 0 showed absence of the nephrogenic zone, lack of cortico-medullary patterning, and the formation of multiple epithelial cysts (Fig. 2A-F). In line with the histological observations, immunofluorescence staining demonstrated that the UB^Hdac1,2−/− neonates completely lack Six2-positive and Pax2-positive cells (Fig. 2G-J).

**UB^Hdac1,2−/− kidneys exhibit stunted UB growth and branching**

To begin to define the embryological mechanisms leading to this phenotype, we monitored UB morphogenesis in a real-time manner in vivo and in vitro, taking advantage of the GFP fluorescence in the UB tissue driven by the Hoxb7 promoter. UB^Hdac1,2−/− mice exhibited attenuated UB branching as early as E13.5 (Fig. 3A), and started to show degeneration of the UB tissue after 1-2 days in culture (Fig. 3B). Previous studies have shown that Hdac1 and Hdac2 regulate apoptosis and proliferation in a wide range of cells. To examine whether increased apoptosis and decreased proliferation contribute to the observed defects in UB branching morphogenesis, we examined the status of cell apoptosis and proliferation at E13.5, when the abnormal UB branching is first noted. Quantification of active caspase 3 (aCasp3)-positive cells revealed a significant increase in the number of apoptotic UB cells in UB^Hdac1,2−/− versus wild-type kidneys at E13.5 (Fig. 4A-E). Consistently, staining of phospho-histone γH2AX, a marker of DNA double-strand breaks, showed that UB^Hdac1,2−/− kidneys exhibited increased DNA damage in UB cells (Fig. 4F-I). Analysis of phospho-histone H3 (pH3), a marker of mitosis, revealed that the rate of cell proliferation was decreased by 37.5% in UB^Hdac1,2−/− relative to wild-type kidneys (Fig. 4J-N). This is consistent with the known pro-proliferative functions of Hdac1 and Hdac2. Taken together, these results indicated that Hdac1 and Hdac2 are crucial for UB cell growth, survival and branching morphogenesis.

**Genome-wide transcriptome analysis of UB^Hdac1,2−/− kidneys**

To further elucidate the developmental pathways regulated by Hdac1 and Hdac2, we carried out a genome-wide microarray analysis on RNA samples extracted from wild-type and UB^Hdac1,2−/− kidneys at E13.5. The raw and analyzed data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE35432. The results revealed that 496/41,000 probes (∼1.2%) are significantly altered in UB^Hdac1,2−/− kidneys (by ≥1.4-fold, P<0.05, n=4), of which 226 transcripts (0.55%) were upregulated.
(range 1.4- to 7.5-fold) and 270 (0.66%) downregulated (range 1.4- to 3.8-fold) (Fig. 5A; see GSE35432).

To analyze whether certain pathways or biological processes are especially sensitive to the loss of Hdac1 and Hdac2 in the UB cells, Ingenuity Pathway Analysis (IPA) was performed on the differentially expressed transcripts. This analysis indicated that the most significantly enriched genes participate in: (1) cell morphology; (2) cellular growth and proliferation; (3) cellular development; (4) cell death and survival and (5) cellular movement (Fig. 5B). The most affected canonical pathways include: (1) basal cell carcinoma signaling; (2) Wnt/β-catenin signaling; (3) sonic hedgehog signaling; (4) human embryonic stem cell pluripotency and (5) tight junction signaling (Fig. 5C). The complete list of genes for each category and pathway is shown in supplementary material Tables S1 and S2.

Further analysis using the Biological Networks Gene Ontology (BiNGO) tool revealed that many genes involved in: (1) tube development; (2) Wnt receptor signaling pathway; (3) ureteric bud development; (4) cell-cell adhesion; (5) kidney development and (6) positive regulation of cell proliferation are downregulated in UB
\(^{Hdac1,2−/−}\) kidneys (Table 1). Interestingly, we found decreased expression of a group of cytokeratins, including Krt7, Krt8, Krt18, Krt19 and Krt23 (Table 2), which is consistent with our immunostaining results using a pan-cytokeratin antibody (Fig. 1I-N). Cytokeratins, the largest intermediate filament protein group, are recognized as peptide fingerprints for the classification of epithelial cells and are implicated in cytoplasmic organization and cellular communication. In embryonic kidneys, Krt8 and Krt18 are specifically expressed in the ureteric epithelium, whereas Krt23 is specifically expressed in the UB tips.

We validated the microarray results by quantitative real-time PCR (qPCR) and in situ hybridization (ISH) of known developmental regulators in the E13.5 kidney. No change was observed in the mRNA expression of Bmyc, cRet, Wnt11, Etv4, Emx2 and Six2 between UB
\(^{Hdac1,2−/−}\) and wild-type kidneys (note that Six2 was downregulated later at P0; Fig. 1). By contrast, Axin2, Shh, Tcf7, Wnt4 and Lef1 were downregulated, and the stromal gene Meis1 was upregulated, confirming our microarray results (Fig. 5D and Fig. 6). It should be noted that qPCR and especially ISH demonstrated a significant decrease of Wnt7b and Wnt9b, whereas the microarray failed to detect this decrease. This is likely to be a sensitivity issue: microarray of whole kidney RNA is not a sensitive approach to detect focal changes in gene expression in the ureteric epithelium.

β-catenin is downregulated in the UB cells of UB
\(^{Hdac1,2−/−}\) kidneys

β-catenin is a multifunctional protein that plays a crucial role in UB branching morphogenesis as well as nephrogenesis. One role of β-catenin is to coordinate cell-cell adhesion through the formation
of adherens junctions with E-cadherin and α-catenin. Another function of β-catenin is to regulate gene transcription, primarily through interactions with the Tcf/Lef family of transcription factors. This function is under the control of the canonical Wnt signaling pathway. Either deletion or overexpression of β-catenin in UB cells leads to kidney hypoplasia (Bridgewater et al., 2008, 2011; Marose et al., 2008). Hdac1 and Hdac2 have been reported to regulate β-catenin in epidermal progenitor cells and oligodendrocytes. In epidermal progenitor cells, Hdac1 and Hdac2 are required for the elevation of β-catenin during hair follicle fate acquisition. Epidermis that is deficient of Hdac1 and Hdac2 displays uniform, low-level expression of β-catenin (LeBoeuf et al., 2010). Conversely, deletion of Hdac1 and Hdac2 in oligodendrocytes results in the stabilization and nuclear translocation of β-catenin, leading to activation of the canonical Wnt/β-catenin pathway (Ye et al., 2009). Our immunofluorescence results showed that β-catenin is dramatically decreased in the UB cells of UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys at E13.5 and thereafter (Fig. 7). These data are consistent with our observation made above that Wnt signaling is repressed in UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys.

To exclude the possibility that we missed an initial activation of Wnt signaling upon loss of Hdac1 and Hdac2 in our animal model, we examined the acute response of cultured embryonic kidneys (E13.5) to HDAC inhibitor, using Axin2, a direct target of canonical Wnt signaling, as a read-out. As indicated by ISH, Axin2 is expressed at a high level in the UB cells and at a low level in the surrounding mesenchymal cells (Fig. 8A). As expected, Axin2 is rapidly induced by the GSK3β inhibitor LiCl, which stabilizes β-catenin and thus activates the canonical Wnt signaling pathway (Fig. 8A). We found that a 6-h treatment with HDAC inhibitor substantially decreases the mRNA level of Axin2 with or without LiCl (Fig. 8B,C). By contrast, expression of cRet is not altered (Fig. 8D). These results suggest that the repression of canonical Wnt signaling in the UB cells of UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys is likely to be a direct rather than secondary effect. As discussed below and shown in supplementary material Table S5, several genes in the Wnt pathway are regulated by Hdac1 and Hdac2 and are also directly bound by p53 in the developing kidney, linking HDAC-Wnt to p53.

**Deletion of Hdac1 and Hdac2 in the ureteric cells leads to p53 hyperacetylation**

At least two studies have shown that Hdac1 and Hdac2 are responsible for deacetylation of p53 (Trp53) in epidermal progenitor cells (LeBoeuf et al., 2010) and oocytes (Ma et al., 2009). We examined UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys for evidence of p53 hyperacetylation. We found a ∼7-fold elevation in the level of total p53 compared with wild-type kidneys: WT 0.54±0.21% versus UB<sup>Hdac1,2<sup>−/−</sup></sup> 3.93±0.77% apoptotic ureteric cells; n=5, *P=0.0029 (t-test), error bars indicate s.e.m. Note that the baseline of apoptosis in wild-type kidneys is very low. (F-I) Representative images showing the induction of DNA damage (phospho-histone γH2AX immunostaining) in UB cells of UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys. (J-M) Proliferating cells were identified using an anti-phospho-histone H3 (pH3) antibody. (N) Quantitative analysis showing that the UB cell proliferation rate, quantitated as the percentage of pH3-positive among total UB cells, was decreased by ∼37.5% in UB<sup>Hdac1,2<sup>−/−</sup></sup> kidneys; WT 6.78±1.00% versus UB<sup>Hdac1,2<sup>−/−</sup></sup> 4.24±0.31% proliferating ureteric cells; n=4, *P=0.0047 (t-test), error bars indicate s.e.m.
Acetylation is a key determinant of p53 function by increasing protein stability, DNA binding affinity and transcriptional activity (Brooks and Gu, 2011; Meek and Anderson, 2009). Previous studies in our laboratory demonstrated that p53 is a key renal development regulator that controls cell proliferation, differentiation and apoptosis pathways (Hilliard et al., 2011, 2014; Saifudeen et al., 2002, 2009). Gain-of-function experiments showed that excessive p53 levels mediated by deletion of \( Mdm2 \) in the UB or MM led to bilateral renal dysplasia, which could be rescued by concurrent removal of p53 (Hilliard et al., 2011, 2014). We therefore asked

Fig. 5. Aberrant gene expression in UB\(^{Hdac1,2^{-/-}}\) kidneys. (A) Microarray analysis of gene expression in UB\(^{Hdac1,2^{-/-}}\) kidneys at E13.5. A small subset of genes (1.2% of 41,000 probes) are significantly altered (>1.4-fold, \( P<0.05 \), \( n=4 \) experiments) in UB\(^{Hdac1,2^{-/-}}\) kidneys. The heat maps display gene expression by log2-transformed fold change (black=0); green indicates lower expression, red indicates higher expression. (B) Molecular functions analysis was performed with IPA. Significantly (\( P<0.05 \)) enriched biological processes are shown. (C) IPA depicts the most affected canonical pathways in UB\(^{Hdac1,2^{-/-}}\) kidneys. Numbers above bars indicate number of altered genes. (D) Validation of microarray results by real-time PCR. Data are presented as mean±s.e., \( n\geq3 \), *\( P<0.05 \) (\( t \)-test).
increases in the levels of p53 acetylated at lysine 370, 379 and function in UB cells. Immunofluorescence revealed marked

Table 1. Selected functional categories of genes downregulated in UB<sup>Hdac1,2</sup>−/− kidneys

<table>
<thead>
<tr>
<th>GO ID</th>
<th>P-value</th>
<th>Description</th>
<th>Genes in test set</th>
</tr>
</thead>
<tbody>
<tr>
<td>35295</td>
<td>3.91E–09</td>
<td>Tube development</td>
<td>Gsta3, Bmp2, Foxa1, Gja1, Hp, Calb1, Shh, Epcam, Fgr, Wnt4, Arg2, Tgm2, Pth1, Hhip, Adams2</td>
</tr>
<tr>
<td>16055</td>
<td>1.82E–08</td>
<td>Wnt receptor signaling pathway</td>
<td>Wnt2, Wnt10a, Fzd10, Wnt4, Tcf7, Left1, Fzb, Axin2, Apccd1, Shh</td>
</tr>
<tr>
<td>1657</td>
<td>2.65E–07</td>
<td>Ureteric bud development</td>
<td>Epcam, Gsta3, Wnt4, Bmp2, Arg2, Calb1, Shh, Mia, Amig2a, Pvr4, Cdh16, Cldn3, Pkd1, Cldn1, Left1, Thy1, Col4a4, Wnt4</td>
</tr>
<tr>
<td>16337</td>
<td>1.34E–06</td>
<td>Cell-cell adhesion</td>
<td>Pkd1, Shh, Wnt2, Cyp7b1, Fos1, Bic, Tgm2, Avpr1a, Shh</td>
</tr>
<tr>
<td>1822</td>
<td>1.23E–02</td>
<td>Kidney development</td>
<td>Pkd1, Shh, Wnt2, Cyp7b1, Fos1, Bic, Tgm2, Avpr1a, Shh</td>
</tr>
<tr>
<td>8284</td>
<td>1.05E–02</td>
<td>Positive regulation of cell proliferation</td>
<td>Pkd1, Shh, Wnt2, Cyp7b1, Fos1, Bic, Tgm2, Avpr1a, Shh</td>
</tr>
</tbody>
</table>

whether Hdac1 and Hdac2 modulate p53 acetylation as well as its function in UB cells. Immunofluorescence revealed marked increases in the levels of p53 acetylated at lysine 370, 379 and 383 (corresponding to lysine 373, 382 and 386 in human, respectively) in the ureteric epithelium of UB<sup>Hdac1,2</sup>−/−/− kidneys at E13.5 (Fig. 9A-D). As anticipated, we also observed an accumulation of total p53 protein in the ureteric epithelium (Fig. 9E,F).

**Germline deletion of p53 partially rescues kidney development in UB<sup>Hdac1,2</sup>−/− mice**

Considering the observed upregulation of p53 in Hdac1/Hdac2-deficient UB cells, we hypothesized that p53 mediates the renal defects observed in UB<sup>Hdac1,2</sup>−/−/− mice, and employed a genetic rescue approach that involved crossing the Hoxb7-Cre<sup>tg/lo</sup>; Hdac1<sup>fl/fl</sup>; Hdac2<sup>fl/fl</sup> mice to p53<sup>−/−</sup> mice. Gross and histological analyses of the kidney at P0 revealed that removal of p53 on the UB<sup>Hdac1,2</sup>−/− background partially rescued kidney development in a gene dosage-dependent manner (Fig. 10). Remarkably, Mendelian proportions of UB<sup>Hdac1,2</sup>−/− p53<sup>−/−</sup> and UB<sup>Hdac1,2</sup>−/− p53<sup>−/−</sup> mice were retrieved at P30, indicating that elimination of one allele of p53 is sufficient to rescue the early postnatal lethality of UB<sup>Hdac1,2</sup>−/−/− mice (Table 3). Thus, although a proportion of UB<sup>Hdac1,2</sup>−/− p53<sup>−/−</sup> and UB<sup>Hdac1,2</sup>−/− p53<sup>−/−</sup> mice exhibited no overt histological rescue, a measurable improvement in renal survival must occur in these mice that allows their survival for at least 30 days. Remarkably, two of the 11 UB<sup>Hdac1,2</sup>−/− p53<sup>−/−</sup> mice were still alive and fertile at 13 months of age and their kidneys exhibited relatively normal histology (supplementary material Fig. S3A-D). These results suggest that hyperacetylation of p53 upon loss of Hdac1 and Hdac2 is one of the important mediators of the congenital renal dysgenesis observed in UB<sup>Hdac1,2</sup>−/−/− mice.

To begin to identify the potential targets of p53 leading to the renal dysgenesis, we cross-reference our UB<sup>Hdac1,2</sup>−/−/− microarray data with p53 ChIP-seq in E15.5 mouse kidneys (Li et al., 2013). Among the 496 altered genes in E13.5 UB<sup>Hdac1,2</sup>−/−/− kidneys, 121 are bound by p53 in their promoter regions (supplementary material Table S3). The Hdac1/2-regulated and p53-bound genes include Lef1, Axin2, Tcf7 (Wnt signaling), Ptc1 (Shh signaling), several pro-apoptosis and cell cycle regulatory genes, kinesin family members (important for ciliary function), calbindin and keratin genes. IPA revealed that the top five molecular and cellular functions regulated by these genes are: (1) cell death and survival; (2) molecular transport; (3) cellular growth and proliferation; (4) cellular development and (5) cell cycle (supplementary material Table S4); and the top five affected canonical pathways are: (1) Vdr/RXR activation; (2) neuroprotective role of Thop1 in Alzheimer’s disease; (3) Wnt/β-catenin signaling; (4) protein kinase A signaling and (5) sonic hedgehog signaling (supplementary material Table S5 and Fig. S4). Hypergeometric distribution analysis demonstrated that cell death and survival genes are highly over-represented (51 of 121 genes, P=0.00037).

Based on the above analyses, we further examined how deletion of p53 affected UB cell survival in UB<sup>Hdac1,2</sup>−/−/− mice. Immunostaining results demonstrated that UB<sup>Hdac1,2</sup>−/−/− p53<sup>−/−</sup> mice had a similar increase in γH2AX-positive UB cells, whereas three of four UB<sup>Hdac1,2</sup>−/−/− p53<sup>−/−</sup> kidneys showed fewer aCasp3-positive UB cells, when compared with UB<sup>Hdac1,2</sup>−/−/− kidneys at E14.5 (4.73±0.47% UB<sup>Hdac1,2</sup>−/−/−; 2.73±0.35% UB<sup>Hdac1,2</sup>−/−/−; p53<sup>−/−</sup> kidneys versus 2.73±0.35% UB<sup>Hdac1,2</sup>−/−/−; p53<sup>−/−</sup> apoptotic ureteric cells, P=0.0249) (Fig. 10C,D).

**Direct effects of HDAC inhibition on cell growth and survival**

To test more directly the cell-autonomous role of p53 hyperacetylation upon loss of HDAC activities, we treated HCT116 p53<sup>(TP53)</sup>+/+ and p53<sup>−/−</sup> isogenic human colon cancer cells with the class I-specific HDAC inhibitor MS-275, which

Table 2. Selected mRNA transcripts decreased in UB<sup>Hdac1,2</sup>−/− kidneys

<table>
<thead>
<tr>
<th>Probe set ID</th>
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<th>Gene name</th>
<th>Fold change</th>
<th>P-value</th>
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<tbody>
<tr>
<td>A_52_P49014</td>
<td>Shh</td>
<td>Sonic hedgehog and hepatic disease 1</td>
<td>-2.90</td>
<td>1.24E–05</td>
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<tr>
<td>A_51_P287100</td>
<td>Cdh16</td>
<td>Cadherin 16</td>
<td>-2.82</td>
<td>2.18E–07</td>
</tr>
<tr>
<td>A_51_P417643</td>
<td>Foxa1</td>
<td>Forkhead box A1</td>
<td>-1.84</td>
<td>9.52E–04</td>
</tr>
<tr>
<td>A_51_P335801</td>
<td>Calb1</td>
<td>Calbindin 1</td>
<td>-1.73</td>
<td>6.72E–06</td>
</tr>
<tr>
<td>A_51_P171616</td>
<td>Wnt10a</td>
<td>Wingless-type MMVT integration site 10a</td>
<td>-1.66</td>
<td>3.68E–05</td>
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<td>A_52_P377941</td>
<td>Pkhd1</td>
<td>Polycystic kidney</td>
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<td>A_52_P61864</td>
<td>Wnt2a</td>
<td>Wingless-type MMVT integration site 2a</td>
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<td>2.56E–03</td>
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<td>A_52_P244702</td>
<td>Tcf7</td>
<td>Transcription factor 7</td>
<td>-1.43</td>
<td>6.24E–03</td>
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<td>A_51_P501145</td>
<td>Left1</td>
<td>Lymphoid enhancer binding factor 1</td>
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<td>A_52_P54176</td>
<td>Axin2</td>
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<td>A_51_P130475</td>
<td>Wnt4</td>
<td>Wingless-type MMVT integration site 4</td>
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<td>A_51_P312348</td>
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<td>Krt23</td>
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preferentially inhibits HDAC1, 2 and 3. Light microscopy analysis revealed that MS-275 induces notable growth arrest and cell death in both p53+/− and p53−/− cells, although p53−/− cells are clearly more resistant to MS-275 than p53+/− cells (supplementary material Fig. S5A).

Next, we quantified the status of the cell cycle and of apoptosis using flow cytometry. We found that the anti-proliferative effects of MS-275 are not dependent on p53 function in HCT116 cells. MS-275 induced G1 and G2 phase arrest in both cell lines, with a decrease in S-phase cells to similar degrees (supplementary material Fig. S5B,D and Table S6), consistent with a previous report that p53 is dispensable for cell cycle arrest in mouse embryonic fibroblasts deficient for Hdac1 and Hdac2 (Wilting et al., 2010). By contrast, p53+/− cells underwent significantly less apoptosis than p53−/− cells (3.52% versus 6.18%, respectively) after a 48-h treatment with 10 µM MS-275 (supplementary material Fig. S5C,E), indicating that MS-275 induces apoptosis partially through p53 in HCT116 cells. Western blot analyses confirmed that MS-275 effectively increased the level of acetylated histone H3 and p53 in p53+/− cells and the level of acetylated histone H3 in p53−/− cells (supplementary material Fig. S5F). Moreover, the total level of p53 is also increased in p53−/− cells (supplementary material Fig. S5F). Cleavage of poly (ADP-ribose) polymerase 1 (PARP1) by caspases is a hallmark of apoptosis; it promotes apoptosis by preventing DNA repair under normal conditions or stress. When we compared the HCT116 cell lines, we identified a strong induction of cleaved PARP1 in p53−/− cells but a much weaker induction in p53+/− cells (supplementary material Fig. S5F). These findings are consistent with a recent report from Sonnemann et al. (2014), showing that HDAC inhibition in HCT116 cells is partially dependent on p53.

**DISCUSSION**

This study investigated the functions of Hdac1 and Hdac2 in the ureteric epithelium during kidney development. Our results demonstrate that: (1) Hdac1 and Hdac2 perform redundant yet essential functions in UB branching morphogenesis; (2) Hdac1 and Hdac2 are required for the canonical Wnt signaling pathway in kidney development; and (3) Hdac1 and Hdac2 are required to suppress hyperacetylation of p53 in the ureteric epithelium, which partially accounts for the UB Hdac1,2−/− phenotype.

**Hdac1 and Hdac2 perform redundant yet essential functions in the UB lineage**

Kidney development is dependent on reciprocal epithelial-mesenchymal interactions between the UB and the MM, which drive iterative branching of the UB and subsequent induction of nephrons. Our previous studies showed that pharmacological inhibition of class I and II HDACs by Scriptaid, or of Hdac1, 2 and 3 by MS-275, causes disruptions of the reciprocal signaling pathways, leading to growth arrest and apoptosis. As many HDAC isoforms (e.g. Hdac1, 2 and 3) are expressed in the UB and MM, it was important to determine the distinct functions of individual enzymes in specific cell lineages. In this study, we set out to elucidate the developmental roles of Hdac1 and Hdac2 in the ureteric epithelium using a conditional knockout strategy. Although global deletion of either Hdac1 or Hdac2 leads to a lethal phenotype (Lagger et al., 2002; Montgomery et al., 2007), demonstrating the unique roles of these two enzymes in early embryogenesis, redundancy of Hdac1 and Hdac2 function has been found in many somatic cell types (e.g. oligodendrocytes, Schwann cells, cardiomyocytes, basal cells and adipocytes) (Haberland et al., 2010; Jacob et al., 2011; LeBoeuf et al., 2010; Montgomery et al., 2007; Ye et al., 2009). In line with the previous studies, our results reveal that one allele of either Hdac1 or Hdac2 is sufficient to support normal kidney development, whereas loss of all four alleles leads to renal hypodysplasia and early postnatal lethality.

**Hdac1 and Hdac2 are required for the canonical Wnt signaling pathway in kidney development**

Genome-wide profiling revealed that changes in gene expression are highly specific in UB Hdac1,2−/− mice at E13.5. Only 226 transcripts (0.55%) were upregulated and 270 transcripts (0.66%) were downregulated (using a cut-off of 1.4-fold, P<0.05). This is consistent with the increasing evidence showing that Hdac1 and
Hdac2 selectively control specific gene expression programs in different tissues. For example, deletion of *Hdac1* and *Hdac2* in the heart resulted in dysregulation of only ~1.6% of the transcriptome (Montgomery et al., 2007). Given the apparent cell apoptosis and growth arrest observed, it is not surprising that genes regulating cell death and survival and cellular growth and proliferation are among the most notably dysregulated functional categories. More interestingly, we found that the canonical Wnt signaling pathway is among the most affected pathways in UB*Hdac1,2−/−* kidneys. The canonical Wnt signaling pathway controls both UB branching morphogenesis and nephrogenesis. Specifically, Wnt7b is expressed in the UB trunk and is essential for the establishment of a cortico-medullary axis during later stages of kidney development. In Wnt7b mutant mice, cortical epithelial development is normal but the medullary zone fails to form (Yu et al., 2009). Wnt9b, another member of the Wnt family secreted by the UB trunk epithelium, plays an indispensable role in the activation of Wnt4, which is both necessary and sufficient for the induction of renal vesicles from renal progenitor cells. Wnt9b is also important for the proliferation/renewal of the renal progenitor cells. Genetic deletion of Wnt9b in mice results in failure of nephron induction and premature exhaustion of the progenitor pool (Carroll et al., 2005; Karner

**Fig. 7.** β-catenin is downregulated in UB*Hdac1,2−/−* kidneys. Immunofluorescence shows that β-catenin is initially expressed at normal levels in UB*Hdac1,2−/−* kidneys at E12.5 (A-F), but is decreased at E13.5 (G-L) and E14.5 (M-T). At E14.5, calbindin staining is too weak to label the UB cells in UB*Hdac1,2−/−* kidneys and Hdac2 staining is used instead.
β-GFP-tagged fully elucidated, time-lapse microscopy using photoactivatable mediator of the Wnt signaling pathway (Perez-Moreno and Fuchs, 2011). Our results show significant decreases in Wnt7b, Wnt9b and Wnt4 expression in UB^{Hdac1,2−/−} kidneys at E13.5, associated with the downregulation of a number of Wnt target genes, such as Axin2, Tcf7 and Lef1. Consistent with these findings, immunofluorescence results demonstrate that membrane-associated β-catenin is greatly decreased in the UB cells of UB^{Hdac1,2−/−} kidneys. β-catenin exists in the cell in two pools: membrane associated and cytosolic. On the membrane, as a component of adherens junctions (AJs), β-catenin links cadherins to the cytoskeleton, whereas in the cytoplasm β-catenin is an essential mediator of the Wnt signaling pathway (Perez-Moreno and Fuchs, 2006). Although the interplay between the two pools remains to be fully elucidated, time-lapse microscopy using photoactivatable GFP-tagged β-catenin has revealed that the membrane-associated pool of β-catenin can be internalized together with E-cadherin, accumulates at the perinuclear endocytic recycling compartment (ERC) upon AJ dissociation, and can be translocated into the nucleus upon Wnt pathway activation (Kam and Quaranta, 2009). Therefore, it is conceivable that decreased levels of membrane-associated β-catenin reduce the availability of cytosolic β-catenin and thus lead to the decrease in Wnt signaling in UB^{Hdac1,2−/−} kidneys. Moreover, we observed that a 6-h treatment of cultured embryonic kidney with HDAC inhibitor leads to a prompt reduction of Axin2, a direct target of Wnt, supporting the idea that Hdac1 and Hdad2 function in the activation of Wnt signaling in the embryonic kidney.

It is also of note that Shh, a hedgehog homolog, is one of the most suppressed genes in UB^{Hdac1,2−/−} kidneys. During kidney development, Shh is initially expressed in the distal part of the UB, and then in the urothelium. Shh acts as a paracrine signal to promote mesenchymal cell proliferation, and regulates the pattern of mesenchymal differentiation. Conditional deletion of Shh in the UB results in renal hypoplasia, hydronephrosis and hydrourourter in newborn pups (Yu et al., 2002). However, such a phenotype is not observed in UB^{Hdac1,2−/−} mice. It is likely to be masked by the more severe dysplastic phenotype of UB^{Hdac1,2−/−} kidneys, or the residual level of Shh is still sufficient to support mesenchymal cell proliferation and differentiation. Taken together, our results demonstrate that Hdac1 and Hdad2 play a crucial role in maintaining essential ureteric genes for renal growth and differentiation.

It should be noted that, although it might be difficult to completely dissociate the direct effects of HDAC inactivation from the secondary effects of cell loss due to apoptosis, there are several clues that support the notion that many of the gene expression changes are specific. First, ISH and microarrays were performed at E13.5, prior to the observed degeneration of the UB tree. Several genes, such as Tcf7, Wnt7b, Wnt9b, Axin2 and Shh, were specifically altered. Second, if the effects of Hdad1/2 inactivation on gene expression were generalized or non-specific due to cell death, we would have expected a much more substantial change in the number of genes altered, as opposed to the few percent of differentially expressed genes observed in the study. Third, our results cannot be fully explained by excessive cell death of UB tip cells since the expression of cRet, Wnt11, Bmyc and Etv4, four specific markers of UB tips, is unaltered in UB^{Hdac1,2−/−} mice.

**Hdac1 and Hdad2 are required to suppress hyperacylation of p53 in the ureteric epithelium**

Although lysine acetylation was originally discovered in histones, these are not the only proteins that can be acetylated. p53 was the first non-histone protein found to be regulated by acetylation (Gu and Roeder, 1997; Luo et al., 2000). The acetylation of multiple lysine residues in the C-terminus (K305, K370, K372, K373, K379 and K383) of p53 increases its stability and activity by preventing ubiquitination and promoting DNA binding (Barjot et al., 2009). Given that HDACs are known to promote p53 acetylation at lysine 370, 379 and 383 in wild-type and UB^{Hdac1,2−/−} kidneys, it was of interest to investigate whether HDAC inhibition could affect p53 expression in UB cells. To this end, we performed experiments using the HDAC inhibitor Scriptaid (hereafter referred to as HDACi).

Inhibition of HDAC activity in cultured metanephroi suppresses Axin2. Paired E13.5 metanephroi were cultured in the presence of 15 mM LiCl or vehicle control (PBS) (A), 2 µg/ml Scriptaid (HDACi) or Nullscript control (B,D), or in the presence of 15 mM LiCl with or without 2 µg/ml Scriptaid (HDACi) (C) for 6 h. Expression of Axin2 (A-C) and cRet (D) was evaluated by whole-mount ISH. The HDAC inhibitor substantially decreases the Axin2 mRNA level, with or without LiCl, whereas cRet is unaffected.

**Fig. 8. Inhibition of HDAC activity in cultured metanephroi suppresses Axin2.** Paired E13.5 metanephroi were cultured in the presence of 15 mM LiCl or vehicle control (PBS) (A), 2 µg/ml Scriptaid (HDACi) or Nullscript control (B,D), or in the presence of 15 mM LiCl with or without 2 µg/ml Scriptaid (HDACi) (C) for 6 h. Expression of Axin2 (A-C) and cRet (D) was evaluated by whole-mount ISH. The HDAC inhibitor substantially decreases the Axin2 mRNA level, with or without LiCl, whereas cRet is unaffected.
K381, K382 and K386) and DNA-binding domain (K164) of human p53 is significantly enhanced in response to stress and required for its stabilization, transcriptional activation and transcription-independent function in apoptosis (Brooks and Gu, 2011). p53 in which all eight lysine residues are substituted (8KR) loses the ability to mediate cell cycle arrest and apoptosis (Tang et al., 2008). There is also evidence that HDAC1 is recruited to p53 by an MDM2-containing protein complex, and HDAC1-mediated deacetylation of p53 is required for degradation of p53 (Ito et al., 2002).

Fig. 10. Elimination of p53 partially rescues kidney development in UB\textsuperscript{Hdac1,2−/−} mice. (A) Representative images of kidneys from newborn mice of the indicated genotypes. Whole-mount (top), transverse sections (middle) and higher magnification (40x, bottom) are shown. (B) Quantification of the renal phenotype of mice of the indicated genotypes, as illustrated in A. The degree of morphological rescue was judged by microscopy and classified as: significant rescue, where kidneys are notably larger and form fewer cysts than UB\textsuperscript{Hdac1,2−/−} p53\textsuperscript{+/+}; moderate rescue, where kidneys (either unilateral or bilateral) are not notably larger but form fewer cysts; no overt rescue, where there is no obvious difference to UB\textsuperscript{Hdac1,2−/−} p53\textsuperscript{+/+} kidneys. (C) Quantitative analysis of UB cell apoptosis (aCasp3 immunostaining) as a percentage of total UB cells indicates that there are fewer apoptotic cells upon p53 deletion from UB\textsuperscript{Hdac1,2−/−}. (D) By contrast, UB\textsuperscript{Hdac1,2−/−} p53\textsuperscript{+/−} mice show a similar elevation in γH2AX-positive UB cells to UB\textsuperscript{Hdac1,2−/−} mice. (C,D) Error bars denote means±s.e.m.
and Hdac2 genes, singly or in combination, specifically in the UB epithelium. For the rescue experiment, p53+/− mice (Jackson Laboratory) were first crossed to the Hdac1flox/flox; Hdac2flox/flox mice, and then further crossed to Hoxb7-CreEGFP transgenic mice to remove p53 on the UBHdac1,2−/− background.

### Histology and immunohistochemistry
Kidneys were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 μm. Histology analyses were performed by standard periodic acid-Schiff (PAS) staining and Hematoxylin and Eosin (H&E) staining. Immunofluorescence was performed as previously described (Chen et al., 2011). Primary antibodies and working concentrations are listed in supplementary material Table S7. The peroxidase-based Vectastain ABC Elite Kit (Vector Laboratories) was used for DAB detection.

### Organ culture
Embryonic kidneys were aseptically micro-dissected from timed-pregnant mice and cultured on polycarbonate Transwell filters (0.4 μm pore size, Corning Costar) over medium [DMEM/F-12 containing 10% fetal bovine serum (FBS)] at 37°C and 5% CO2.

### RNA extraction
Kidneys were harvested at E13.5 and stored in RNAlater RNA stabilization reagent (Qiagen). Wild-type and UBHdac1,2−/− kidneys were each divided into four random pools (n=4). Total RNA was then isolated using the RNeasy Mini Kit (Qiagen).

### Genome-wide microarray analysis
Microarray analysis was performed according to established protocols (Schanstra et al., 2007). Briefly, fluorescently labeled cRNA was generated from 0.5 μg total RNA in each reaction using the Agilent Fluorescent Direct Label Kit and 1.0 mM Cyanine 3− or 5−labeled dCTP (PerkinElmer). Hybridization was performed using the Oligonucleotide Microarray Hybridization and In Situ Hybridization Plus Kit (Agilent). The labeled cRNA was hybridized to Agilent 44K whole mouse genome oligonucleotide microarray (containing ~41,000 probes) as previously described (Schanstra et al., 2007). The arrays were scanned using a dual-laser DNA microarray scanner (Agilent). The data were then extracted from images using Feature Extraction software 6.1 (Agilent). Microarray data are available at GEO under accession number GSE35432.

### Data analysis
MultiExperiment Viewer v4.9 software was used to generate lists of genes differentially expressed between wild-type and UBHdac1,2−/− kidneys, using P≤0.05 and a minimum 1.4-fold change in gene expression. Genes were classified according to their function using IPA software and BINGO classification systems as previously described (Chen et al., 2011).

### qPCR
Quantitative real-time PCR (qPCR) was performed using the One-Step Brilliant Quantitative RT-PCR Master Mix Kit (Applied Biosystems). Real-time PCR reaction mix contained 200 nm forward primer, 200 nm reverse primer, and 60 ng total RNA. Relative levels of mRNA were normalized to Gapdh. The primers used for qPCR are listed in supplementary material Table S8.

### Whole-mount in situ hybridization (ISH)
ISH was performed using digoxigenin-labeled antisense probes on kidney tissue fixed with 4% paraformaldehyde as previously described (Chen et al., 2011).

### Cell culture and treatments
HCT116 p53+/− and p53−/− cells were obtained from Dr Hua Lu (Tulane University, New Orleans, LA, USA, and Johns Hopkins University Cell Center, Baltimore, MD, USA). Cells were grown in high-glucose DMEM with
stable glutamine supplemented with 10% FBS and 10 mg/ml antibiotics (penicillin and streptomycin), under 5% CO₂ and saturated moisture. Cells were treated with vehicle control DMSO or 10 µM MS-275 for 24, 48 or 72 h.

### Analysis of cell cycle and apoptosis using flow cytometry
Cells were treated with vehicle control DMSO or 10 µM MS-275 for 48 h. At the time of harvesting, cells were digested with 0.05% trypsin and resuspended in phosphate-buffered saline (PBS). For cell cycle analysis, 2×10^5 cells were stained with 50 µg/ml propidium iodide (PI) using the Coulter DNA Prep Reagents Kit (Beckman Coulter). Cells were then analyzed using a Beckman Coulter FC500 flow cytometer and CXP software (Beckman Coulter) for acquisition and analysis. The cell cycle distribution was further analyzed by ModFit LT 3.0 software (Verity Software House).

### Western blot
Whole-cell proteins were extracted using RIPA buffer (Invitrogen) and nuclear proteins were extracted using the Nuclear Extract Kit (Active Motif). Primary antibodies and working concentrations are listed in supplementary material Table S7.

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### Competing interests
The authors declare no competing or financial interests.

### References


