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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Histone deacetylases 1 and 2 regulate the transcriptional programs of nephron progenitors and renal vesicles

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ABSTRACT

Nephron progenitor cells (NPCs) are Six2-positive metanephric mesenchyme cells, which undergo self-renewal and differentiation to give rise to nephrons until the end of nephrogenesis. Histone deacetylases (HDACs) are a group of epigenetic regulators that control cell fate, but their role in balancing NPC renewal and differentiation is unknown. Here, we report that NPC-specific deletion of *Hdac1* and *Hdac2* genes in mice results in early postnatal lethality owing to renal hypodysplasia and loss of NPCs. HDAC1/2 interact with the NPC renewal regulators Six2, Osr1 and Sall1, and are co-bound along with Six2 on the *Six2* enhancer. Although the mutant NPCs differentiate into renal vesicles (RVs), *Hdac1/2* mutant kidneys lack nascent nephrons or mature glomeruli, a phenocopy of *Lhx1* mutants. Transcriptional profiling and network analysis identified disrupted expression of *Lhx1* and its downstream genes, *Dll1* and *Hnf1a*/*4a*, as key mediators of the renal phenotype. Finally, although HDAC1/2-deficient NPCs and RVs overexpress hyperacetylated p53, *Trp53* deletion failed to rescue the renal dysgenesis. We conclude that the epigenetic regulators HDAC1 and HDAC2 control nephrogenesis via interactions with the transcriptional programs of nephron progenitors and renal vesicles.

KEY WORDS: Epigenetics, Histone deacetylase, Kidney development, Nephron progenitors

INTRODUCTION

Kidney development requires precise integration of various progenitor cell populations. In ∼1/500 births, some abnormality occurs in kidney development, leading to congenital anomalies of the kidney and urinary tract (CAKUT) (Schedl, 2007). CAKUT accounts for up to 30% of end-stage renal disease in children less than 4 years of age (North American Pediatric Renal Trials and Collaborative Studies 2008 Annual Report; https://web.emmes.com/study/ped/annlrept/Annual%20Report%20-2008.pdf). Moreover, CAKUT increases the risk of development of hypertension and other cardiovascular diseases in adulthood (Wuhl et al., 2013). The formation of a sufficient number of nephrons is crucial for final kidney function in the adult and requires a delicate balance between nephron progenitor cell (NPC) self-renewal and differentiation. Conversely, unrestrained NPC expansion and arrested differentiation lead to Wilms' tumor, an embryonic tumor of the kidney (Kreidberg and Hartwig, 2008).

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Six2, a homeodomain transcription factor, is a key factor within the kidney metanephric mesenchyme that maintains the NPC pool (Kobayashi et al., 2008; Self et al., 2006). In *Six2* null mice, ectopic renal vesicles (the earliest epithelialized forms of nascent nephrons) develop at the onset of nephrogenesis and the progenitor pool is rapidly lost (Self et al., 2006). Many transcriptional regulators – such as Osr1, WT1 and Sall1/Mi-2b (Chd4)/nucleosome remodeling and deacetylase (NuRD), which function to maintain the NPC pool – display genetic interactions with Six2 (Basta et al., 2014; Denner and Rauchman, 2013; Hartwig et al., 2010; Kanda et al., 2014; Xu et al., 2014). In addition, it has been shown that Six2 regulates self-renewal and commitment of NPCs through sharing gene regulatory networks with Wnt proteins (Park et al., 2012). However, the details of how these networks operate to maintain the multipotency of nephron progenitors are not well understood. This knowledge is necessary to understand mechanisms of nephrogenesis and for therapeutic intervention of kidney diseases.

Recent years have witnessed an expanded awareness of the crucial role of epigenetic mechanisms in health and disease (Egger et al., 2004). During development, epigenetic mechanisms – such as DNA methylation, histone modifications and miRNA biogenesis – program the genome in a particular cell by alteration of chromatin structure and DNA accessibility to the transcriptional machinery. Disruptions of these epigenetic mechanisms can lead to dysregulation of gene function, without altering the DNA sequence itself (Egger et al., 2004). As epigenetic abnormalities depend on the interplay between genes and the environment, they are often phenotypically variable, which fits well with the broad phenotypic spectrum of CAKUT. Therefore, understanding the epigenetic basis of kidney development might provide new insights into the pathological mechanisms of CAKUT and, hopefully, open new avenues to treatment or prevention of CAKUT, through pharmaceutical agents that target epigenetic modifiers. Histone deacetylases (HDACs) are an evolutionarily conserved group of enzymes that remove acetyl groups from histones as well as nonhistone proteins [e.g. p53 (tumor protein p53)]. HDACs regulate gene expression in a highly selective way, and exhibit both repressive and activating effects (Haberland et al., 2009). To date, 18 mammalian HDACs have been identified. HDAC1 and HDAC2 share high sequence identity of ∼83% (de Ruijter et al., 2003) and regulate gene expression as the catalytic core of three major multiprotein co-repressor complexes: Sin3 (Sin3a), NuRD and co-repressor for element-1-silencing transcription factor (CoREST; Rcor2) (Kelly and Cowley, 2013). During embryogenesis, HDAC1 and HDAC2 play both redundant and distinct functions in a tissue-specific manner (Brunmeir et al., 2009; Jacob et al., 2011, 2014; LeBoeuf et al., 2010; Turgeon et al., 2013; Winter et al., 2013; Ye et al., 2009). Our previous studies showed that HDAC activity is required for key developmental pathways regulating overall renal growth and differentiation and ureteric bud (UB) branching (Chen

Development (2018) 145, dev153619. doi:10.1242/dev.153619

et al., 2011, 2015). In the present work, using conditional targeting in Six2^{+} NPCs, we unraveled novel roles of HDAC1/2 in the control of NPC maintenance. Moreover, our findings implicate HDAC1/2 in the regulation of the differentiation program of renal vesicles (RVs) into nascent nephrons. Thus, HDAC1/2 regulate nephron endowment through actions on multiple steps of nephrogenesis.

RESULTS

NPC-specific deletion of *Hdac1* and *Hdac2*

To gain insights into the role of HDAC1/2 in NPC maintenance and differentiation, we crossed *Six2eGFPCre* (*Six2^{GC}*) mice (Kobayashi et al., 2008) with *Hdac1$^{flox/flox}$;Hdac2$^{flox/flox}$* mice (Montgomery et al., 2007). To test the efficacy of Six2-driven Cre-mediated excision, we examined the expression of HDAC1 and HDAC2 proteins in wild-type (WT) and mutant kidneys. As previously reported by our group (Chen et al., 2011), HDAC1/2 are nuclear proteins abundantly expressed in the nephrogenic zone (Fig. 1A,C,E). In *Six2^{GC};Hdac1$^{flox/flox}$;Hdac2$^{flox/flox}$* (herein referred to as HDAC1/2 mutant) mice, HDAC1/2 are not detected in the cap mesenchyme (CM) but are maintained in the surrounding UB branches and stromal cells (Fig. 1B,D,F). In accordance with the key functions of HDAC1/2 in deacetylation of histones and p53, the acetylation levels of H3K9, H4 (K5, K8, K12 and K16), and p53 (K386) are substantially increased in the NPC and derivatives [pretubular aggregates (PTAs) and RVs] of HDAC1/2 mutant kidneys (Fig. 2A-F). Collectively, these results demonstrate the efficient deletion of *Hdac1* and *Hdac2* from the Six2^{+} nephron progenitor pool.

NPC-specific deletion of *Hdac1* and *Hdac2* causes renal hypodysplasia

Mice with NPC-specific double deletion of *Hdac1* and *Hdac2* (all four alleles) were born in normal Mendelian ratios; however, they died soon after birth. At birth, *Hdac1/2* mutant mice exhibited bilaterally small kidneys with full penetrance and there were some obvious petechial hemorrhagic spots on the surface of the mutant kidneys (Fig. 3A-C). Histological examination of mutant kidneys at postnatal day (P) 0 showed small kidney size, absence of the nephrogenic zone, lack of nascent nephrons and glomeruli, and formation of multiple cysts (Fig. 3D-I). *Lotus tetragonolobus* lectin (LTA) staining determined that the majority of cortical renal cysts originate from proximal tubules (LTA-positive tubules, Fig. 1B,D). In contrast, one allele of either *Hdac1* or *Hdac2* is sufficient to ensure nephrogenesis (Fig. 3B,E,H) and survival until adulthood, although these mice show subtle phenotypes including fewer nephrons with variable penetrance (data not shown).

NPC-specific deletion of *Hdac1/2* inhibits cell proliferation but not survival

The defects in nephrogenesis in HDAC1/2 mutant kidneys could have partly resulted from decreased cell proliferation and/or increased apoptosis. Proliferating cell nuclear antigen (PCNA) is a

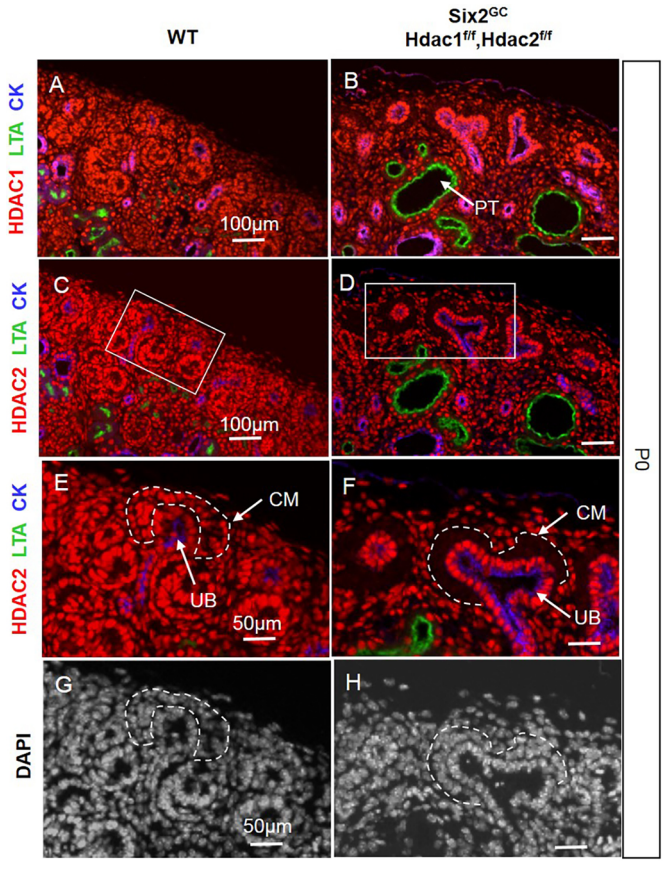

Fig. 1. Deletion of *Hdac1* and *Hdac2* genes in the CM. (A,C,E,G) Consecutive section immunofluorescence (IF) at P0 showing the relatively abundant nuclear expression of HDAC1/2 proteins in the nephrogenic zone within the CM, UB and stroma. (B,D,F,H) Conditional Six2-Cre-mediated deletion of *Hdac1/2* genes results in efficient loss of HDAC1/2 proteins from the CM. Boxes in C and D are shown enlarged in E and F, respectively. The scale bar information is the same in the left-hand and right-hand panels. CK, pancytokeratin; CM, cap mesenchyme; LTA, *Lotus tetragonolobus* lectin; PT, proximal tubule; UB, ureteric bud branches.

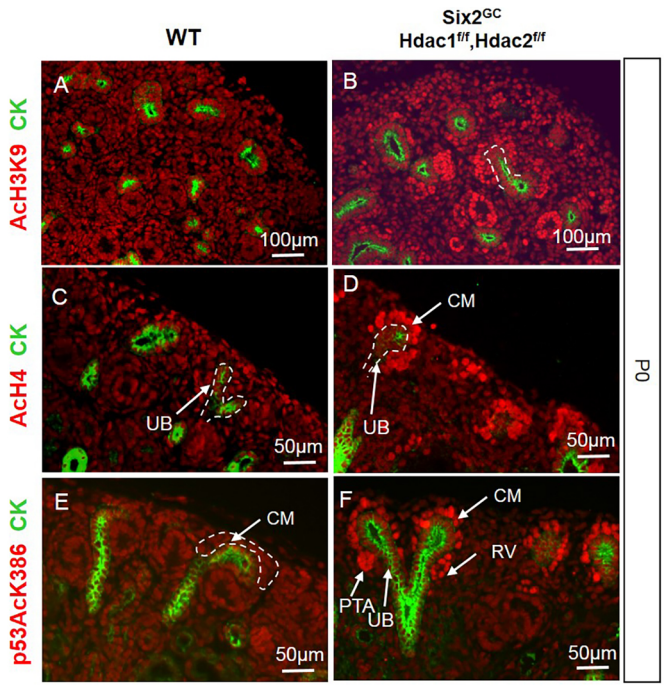

Fig. 2. NPC-specific deletion of *Hdac1* and *Hdac2* causes hyperacetylation of histones H3 and H4 and p53. (A,C,E) AcH3K9, AcH4 and p53AcK386 are expressed at relatively low levels in all cell types of the developing kidney. (B,D,F) In HDAC1/2 mutant kidneys, there is upregulation of acetylated H3K9, H4 and p53 in NPCs and derived nascent tubules. AcH3K9, histone H3 (acetyl K9), AcH4, acetyl-histone H4; CK, pancytokeratin; CM, cap mesenchyme; p53AcK386, acetyl-Lys386 p53; PTA, pretubular aggregate; RV, renal vesicle; UB, ureteric bud branch.

Development (2018) 145, dev153619. doi:10.1242/dev.153619

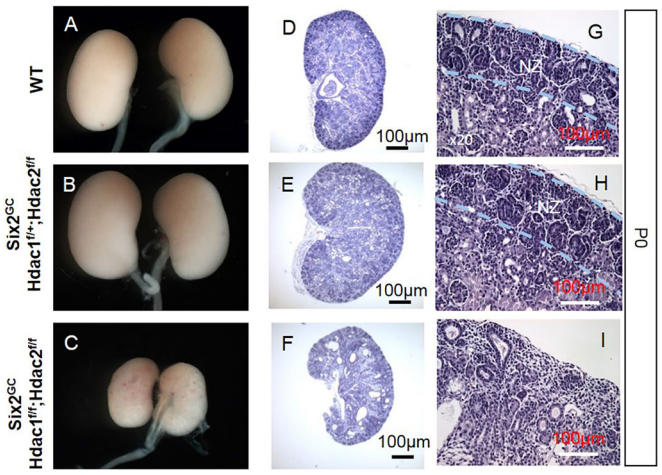

Fig. 3. NPC-specific deletion of *Hdac1* and *Hdac2* causes severe renal hypodysplasia. (A-I) Gross and histological morphology in wild-type (WT) (A,D,G), and conditional compound heterozygous (B,E,H) and homozygous null (C,F,I) HDAC1/2 mutant mice. Six2-Cre-mediated deletion of HDAC1/2 impairs renal growth and patterning owing to loss of the nephrogenic zone (NZ) and cystic tubular degeneration.

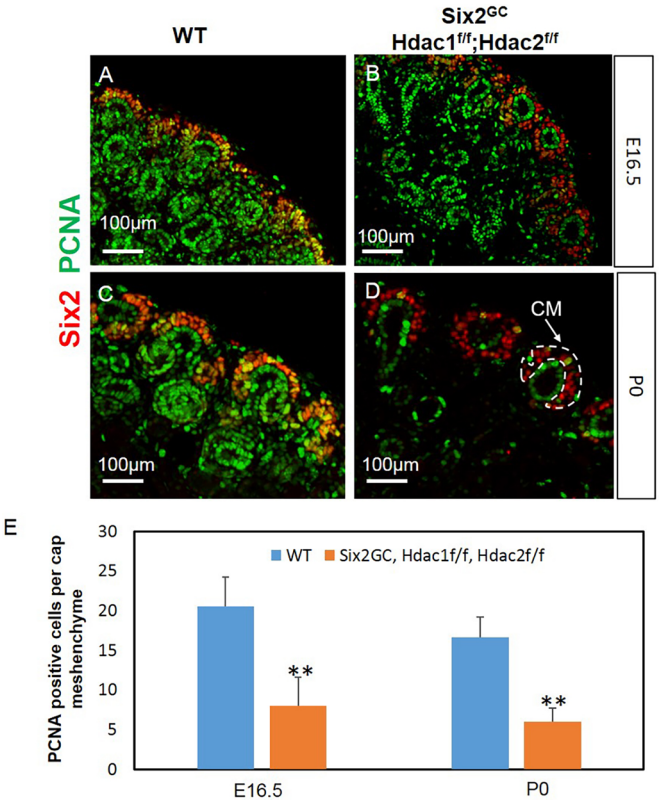

Fig. 4. NPC-specific deletion of *Hdac1* and *Hdac2* inhibits cellular proliferation in the CM and nephrogenic zone. Section IF for PCNA and the NPC-specific marker Six2 at E16.5 (A,B) or P0 (C,D). There is reduction/loss of proliferating cells in the CM and nephrogenic zone in HDAC1/2 mutants compared with controls. Original magnification ×20. (E) Quantification of PCNA staining showed significantly decreased cell proliferation in E16.5 and P0 mutant CM. Data are mean±s.e.m. $^{**}P<0.05$; $n=3$ animals per group.

sliding clamp that serves as a loading platform for many proteins involved in DNA replication and DNA repair (Strzalka and Ziemienowicz, 2011). PCNA protein expression and synthesis is linked with cell proliferation, and PCNA associates with nuclear histone deacetylase activity (Milutinovic et al., 2002). Moreover, cell proliferation defects are commonly found in most HDAC1/2 knockout or knockdown models (Haberland et al., 2009; Kelly and Cowley, 2013). Immunostaining for PCNA at embryonic day (E) 16.5 and P0 revealed that HDAC1 and HDAC2 are essential for proliferation of NPCs and their derivatives in the nephrogenic zone (Fig. 4A-D). Quantitatively, *Hdac1/2* deletion resulted in a ∼75% reduction in the number of proliferating cells per CM ($P<0.05$, $n=3$ per group) (Fig. 4E). We also observed that the remnant Six2 cells seem to form a single cell layer surrounding the UB tips in the mutant CM niches (Fig. 4B, Fig. S1). We do not have an explanation for this unusual observation and are not aware of other mutants exhibiting this abnormality in patterning of the CM around the UB tip. Whether this represents an intrinsic defect in the organization of HDAC1/2 mutant $Six2^{+}$ cells, or results from abnormal organization of the UB tips, remains to be elucidated. We next investigated whether HDAC1/2-deficient NPCs exhibit increased levels of apoptosis. Co-immunostaining of active caspase 3 and Six2, as well as terminal deoxynucleotidyl transferase (rTdT) mediated biotin-dUTP nick end-labeling (TUNEL) assay, at E14.5 (not shown) and P0 showed that deletion of *Hdac1/2* had no obvious effect on cell survival in the NPCs (Fig. S1). Taken together, these results indicate that HDAC1 and HDAC2 are essential for NPC growth, but not survival.

p53 hyperacetylation is not a mediator of renal dysgenesis in HDAC1/2 mutants

In addition to their chromatin modifying activities, HDAC1/2 deacetylate the transcription factor p53. p53 is induced by cell stress via post-translational modifications, including acetylation of its C-terminus by CBP (Crebbp)/p300 (Ep300). Hyperacetylated p53 has been linked to transcriptional activation, which in turn induces cell cycle arrest and/or apoptosis. We therefore tested whether the observed p53 hyperacetylation in NPCs and derivatives resulting from HDAC1/2 deletion contributes to the renal dysgenesis. We generated triple mutant $Six2^{GC};Hdac1^{flox/flox};Hdac2^{flox/flox};p53^{+/-}$ and $Six2^{GC};Hdac1^{flox/flox};Hdac2^{flox/flox};p53^{-/-}$ mouse strains and examined the pups at P0. The results showed that triple mutant kidneys continue to exhibit depletion of NPCs and arrest of tubular differentiation (Fig. S2). In fact, loss of p53 exaggerated renal cystogenesis in this model (Fig. S2A-D). Thus, genetic p53 deletion fails to rescue the HDAC/12 mutant renal phenotype.

NPC-specific deletion of *Hdac1/2* represses the NPC self-renewal genes

We next assessed the molecular phenotype resulting from deletion of *Hdac1/2* in the NPCs. Immunostaining of Six2, Pax2, Sall1 and WT1 and *in situ* hybridization (ISH) of *Osr1*, markers and key regulators of the CM, demonstrated that progenitor gene expression and the NPC pool are dramatically reduced or absent in E14.5, E16.5 and P0 HDAC1/2 mutant compared with WT kidneys (Fig. 5A-N, Fig. S3A,B).

HDAC1/2 interact with NPC regulators and are bound to the *Six2* enhancer

We next investigated whether HDAC1/2 interact biochemically with the NPC regulators Six2, Osr1 and Sall1 in transfected human embryonic kidney (HEK) 293T cells. Immunoprecipitation of either Flag-HDAC1 or Flag-HDAC2 pulled down Myc-tagged Six2 (Fig. 6A). Conversely, immunoprecipitation of Myc-tagged Six2

Development (2018) 145, dev153619. doi:10.1242/dev.153619

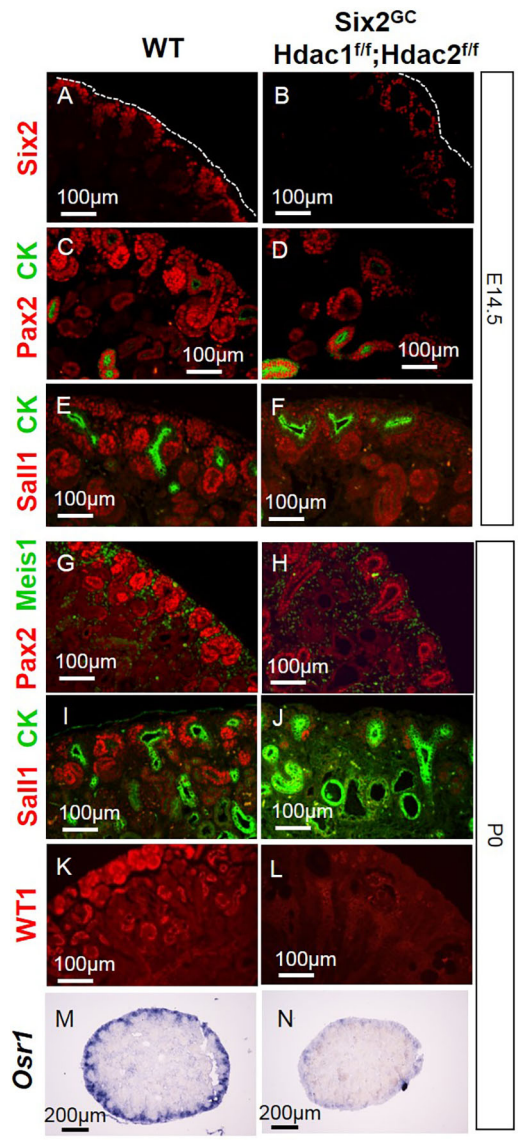

Fig. 5. NPC-specific deletion of *Hdac1* and *Hdac2* depletes nephron progenitors and results in loss of NPC markers. Section IF at E14.5 (A-F) or P0 (G-N) reveals decreased expression of Six2, *Osr1*, Pax2, Sall1 and WT1. Section ISH shows decreases expression of *Osr1* at P0 (M,N) CK, cytokeratin.

pulled down Flag-HDAC1 and Flag-HDAC2 (Fig. 6A). Osr1 acts downstream of and interacts synergistically with Six2 to maintain NPCs (Xu et al., 2014). We tested whether co-expressed Myc-Osr1 is able to interact with transfected Flag-HDAC1 and Flag-HDAC2. Co-immunoprecipitation showed robust interactions of Osr1 with both HDAC1 and HDAC2 (Fig. 6B). Furthermore, immunoprecipitation of Flag-Sall1 pulled down endogenous HDAC1, HDAC2 and transfected Myc-Six2 (Fig. 6C). In addition, we also detected endogenous interaction between Six2 and HDAC1 and HDAC2 in mouse E16.5 kidneys (Fig. 6D), P0 kidneys and E16.5 NPCs (Fig. S4). Of note, immunoprecipitation of HDAC1 pulled down a small amount of endogenous HDAC1 and Six2, whereas HDAC2 immunoprecipitation pulled down HDAC1 and an obviously higher amount of Six2. Collectively, these data suggest that HDAC1 and HDAC2 interact with Six2, Sall1 and Osr1, which are essential players in the balance of NPC self-renewal and differentiation.

Chromatin immunoprecipitation (ChIP) followed by NextGen sequencing in embryonic kidneys revealed that Sall1 and Six2 co-occupy many loci containing genes essential for kidney development, as well as *Sall1* and *Six2* themselves (Kanda et al., 2014). Interestingly, in the *Six2* gene, Sall1 binding sites lie within 500 bp of the Six2 binding site (Kanda et al., 2014). The Six2/Sall1-bound region corresponds to that reported by Park et al., and is located 60 kb upstream of the *Six2* transcription start site (Park et al., 2012). This region directs faithful spatial and temporal expression of a reporter in transgenic mice (Park et al., 2012). We tested whether HDAC1/2 and Six2 are bound to the *Six2* enhancer in NPCs. We isolated NPCs from E16.5 kidneys by magnetic-activated cell sorting (Brown et al., 2015) and performed ChIP-PCR using anti-HDAC1, anti-HDAC2 and anti-Six2 antibodies. The isolated NPCs are highly enriched with Six2 (90%), with minor contamination with stromal cells (Meis1) (Fig. S5). The results showed that HDAC1/2 and Six2 co-occupy the *Six2* enhancer (Fig. 6E). The specificity of the HDAC1 antibody was further validated by ChIP-PCR using positive and negative control primer sets (Fig. S6). Because HDAC1/2 and Six2 proteins interact (Fig. 6A), these findings suggest a model in which Six2 recruits HDAC1/2 to the *Six2* enhancer, and this interaction might be necessary for regulation of *Six2* expression.

HDAC1/2 are required for *Lhx1* gene expression and renal vesicle differentiation

In E14.5-E16.5 HDAC1/2 mutant kidneys, early nephron precursors such as pre-tubular aggregates and RVs form. However, only rare comma- and S-shaped nascent nephrons or proximal tubules were detected (Fig. 7A-F), suggesting that HDAC1/2 are required for the RVs to progress to comma- and S-shaped nephrons and eventually into segmented epithelial nephrons.

Genome-wide transcriptome analysis of whole kidney RNA (see below) revealed reduced expression of *Lhx1* in HDAC1/2 mutant kidneys. Lhx1, also known as LIM-class homeodomain transcription factor 1 (Lim-1), is expressed in the intermediate mesoderm, nephric duct, mesonephric tubules, ureteric bud, pretubular aggregates and RVs (Kobayashi et al., 2005; Pedersen et al., 2005). Lhx1 function is required for patterning and RV maturation into comma- and S-shaped bodies because tubulogenesis is blocked at the RV stage in *Lhx1* null mice (Kobayashi et al., 2005; Pedersen et al., 2005). Accordingly, we examined Lhx1 expression in HDAC1/2 mutant kidneys. In E16.5 WT kidneys, Lhx1 protein is expressed in the RVs and nascent nephrons (Fig. 8A,C). In contrast, Lhx1 expression is abrogated in the HDAC1/2-deficient RVs and nascent nephrons (Fig. 8B,D-F). Interestingly, the few remnant RVs and nascent nephrons observed in the HDAC1/2 mutant kidneys appear to have escaped Cre-mediated recombination (Fig. 8G,H).

NPC-specific deletion of *Hdac1/2* downregulates *Pax8* and *Fgf8* in RVs

During nephrogenesis, *Lhx1* is downstream of *Fgf8*, *Pax8* and *Wnt4*, whereas *Wnt4* lies downstream of *Pax8/Fgf8* and upstream of *Lhx1* (Grieshammer et al., 2005; Kispert et al., 1998; Kobayashi et al., 2005; Narlis et al., 2007; Park et al., 2007; Pedersen et al., 2005; Perantoni et al., 2005; Stark et al., 1994). Section ISH in E16.5 and P0 mice demonstrated that both *Fgf8* and *Pax8* are markedly downregulated in the HDAC1/2 mutant versus control RVs (Fig. 9A-D,G,H). Also, we confirmed abrogated *Lhx1* expression in the mutant RVs (Fig. 9E,F,I,J). In contrast, *Wnt4*

Development (2018) 145, dev153619. doi:10.1242/dev.153619

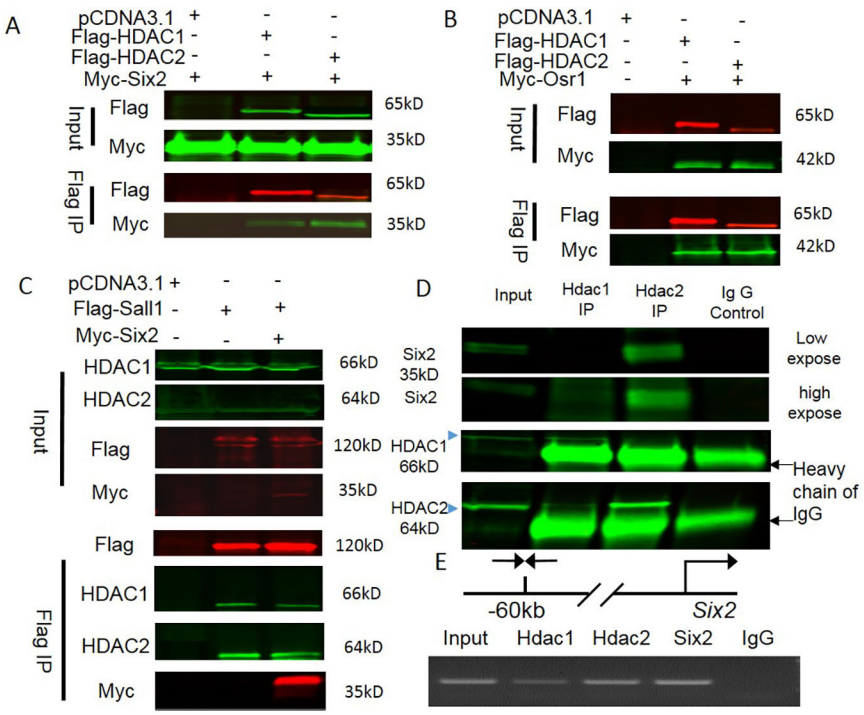

Fig. 6. HDAC1/2 interact with NPC transcriptional regulators and bind the *Six2* enhancer. (A-C) The designed plasmids (+) were transfected into HEK 293T cells and the cell lysates were subject to Flag-tag immunoprecipitation. Flag-HDAC1/2 co-immunoprecipitate with Myc-Six2 (A); Flag-HDAC1/2 co-immunoprecipitate with Myc-Osr1 (B); Flag-Sall1 and Myc-Six2 co-immunoprecipitate with endogenous HDAC1/2 (C). (D) Endogenous interaction between Six2 and HDAC1/2 was detected in E16.5 kidneys. (E) Chromatin immunoprecipitation-PCR showing HDAC1/2 and Six2 co-occupancy of the *Six2* enhancer.

mRNA expression [ISH and quantitative PCR (qPCR)] is maintained in the mutant RVs but not in NPCs (Fig. 10A-C). Thus, loss of *Lhx1* expression in HDAC1/2 mutant RVs is Wnt4 independent, but at least partly caused by decreased expression of *Pax8/Fgf8*.

Interestingly, we found enhanced expression of the canonical Wnt protein target, Lef1, in the stromal mesenchyme surrounding the UB branches as well as in the outer cortical stroma of HDAC1/2 mutant kidneys (Fig. 10D,E). This increase does not appear to be mediated by excess Wnt9b expression in the UB (Fig. S7).

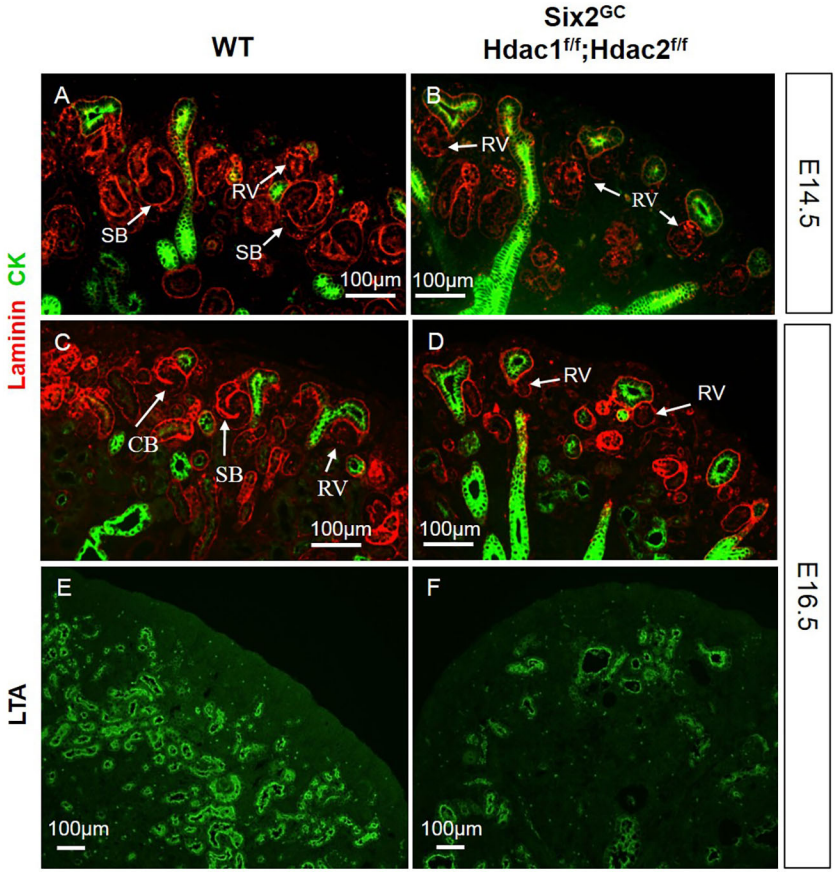

Fig. 7. NPC-specific deletion of *Hdac1* and *Hdac2* arrests nephrogenesis at the RV stage. (A-F) Section IF at E14.5 (A,B) and E16.5 (C-F). Laminin staining outlines the basement membrane of nascent nephrons showing that nephrogenesis is arrested at the RV stage in double HDAC1/2 mutant kidneys as early as E14.5. Few and scattered proximal tubules are formed in mutant kidneys. CK, pancytokeratin; LTA, *Lotus tetragonolobus* lectin; RV, renal vesicle; SB, S-shaped body.

Development (2018) 145, dev153619. doi:10.1242/dev.153619

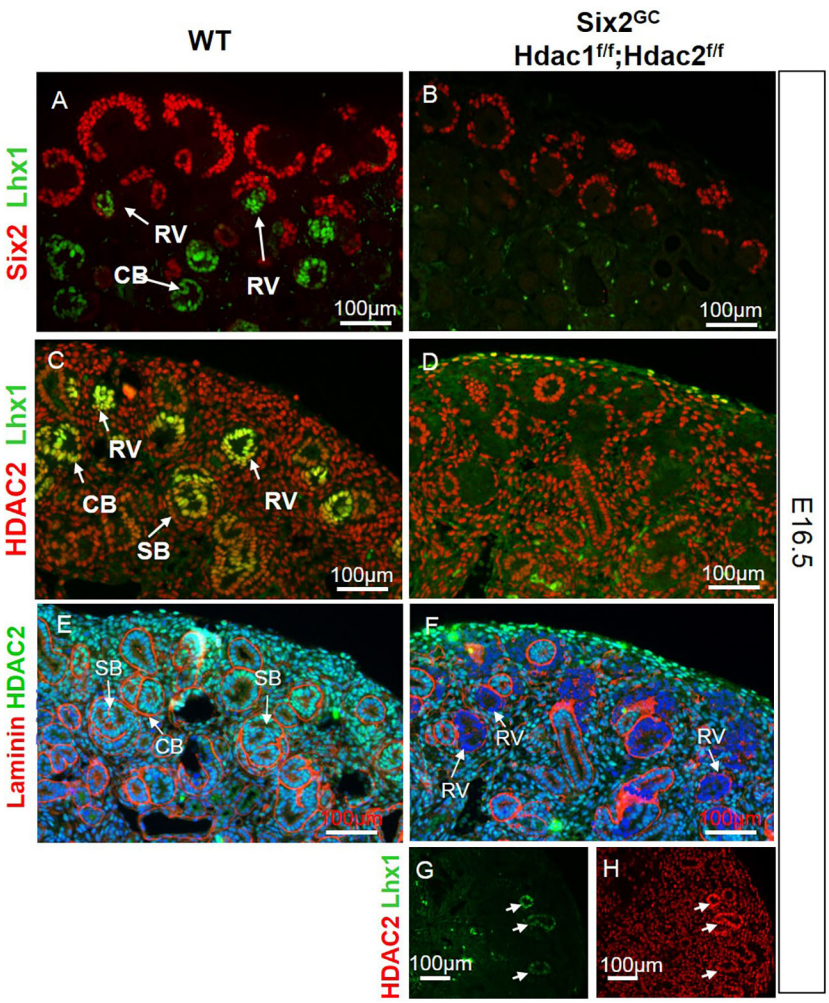

Fig. 8. RVs lacking HDAC1/2 fail to express the transcription factor Lhx1. (A-F) Section IF for Six2/Lhx1 (A,B), HDAC2/Lhx1 (C,D), and laminin/HDAC2 (E,F) showing abrogated Lhx1 protein expression in nascent nephrons of HDAC1/2-mutant kidneys, which correlates spatially with loss of HDAC1/2. (G,H) The few instances in which Lhx1 expression was preserved correlate with nascent nephrons that escaped Cre-mediated excision of HDAC1/2. Arrows indicate the different stages of nascent nephrons: renal vesicle (RV), comma-shaped body (CB) and S-shaped body (SB).

However, because it is difficult to clearly compare the Lef1 levels between WT and mutant NPCs from Lef1 immunostaining alone, we examined the effect of HDAC1 and HDAC2 deletion on *Lef1* gene expression in fluorescence-activated cell sorting (FACS)-isolated Six2-GFP^{+} NPCs from E16.5 WT and HDAC1/2 mutants. NPCs were cultured in differentiation (KO) medium (Brown et al., 2011, 2013) in the presence of 3.5 µM CHIR for 48 h. *Lef1* RNA copy number, quantitated by droplet digital PCR (ddPCR), was not different in CHIR-treated HDAC1/2 mutant and WT NPCs (Fig. 10F). In contrast, *Six2 RNA* copy number was 50% lower in mutant CHIR-treated than in WT CHIR-treated NPCs. Collectively, these findings indicate that loss of HDAC1/2 in NPCs and derived cells activates stromal Wnt signaling, suggesting the presence of intercompartmental crosstalk. Of note, our transcriptome profiling identified multiple stromal genes (*Pbx1*, *Meis1*, *Foxd1*, *Fat4*) that are upregulated in the HDAC1/2 mutant kidneys. Our data also suggest that *Six2* expression is dependent on HDAC1/2 in the setting of activated Wnt signaling.

Transcriptome analysis of whole kidney RNA

To further understand the molecular pathogenesis of the renal phenotype and to elucidate the developmental pathways regulated by HDAC1 and HDAC2, we carried out a genome-wide microarray analysis of RNA samples extracted from E15.5 WT and *Hdac1/2* kidneys. The raw and analyzed data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE84305. The results revealed that 649 transcripts (1.17%) are significantly altered in double-mutant kidneys (≥1.5-fold, P<0.05, n=4 independent experiments), of which 349 (69.24%) were upregulated (range +1.50- to +8.56-fold) and 155 transcripts (30.76%) were downregulated (range −1.50- to −12.40-fold) (Fig. S8A).

To analyze the pathways and biological processes that are sensitive to the loss of HDAC1/2, Ingenuity Pathway Analysis (IPA) was performed on the differentially expressed transcripts. This analysis indicated that the most significantly enriched pathways are concerned with cancer, embryonic development and organ development (Fig. S8B-D). A complete list of genes for each category and pathway is shown in Table S1. Further analysis using the Biological Networks Gene Ontology (BiNGO) tool revealed that many genes involved in kidney development processes are altered in HDAC1/2 mutant kidneys; for example, key factors involved in differentiation of the proximal and distal nephrons, such as *Lhx1* (−1.6-fold), *Hnf1a* (−1.90-fold), *Hnf4a* (−2.85-fold), *Irx1* (−2.346-fold), and the Notch signaling pathway [*Dll1* (−2.18-fold), *Hes5* (−2.24-fold), *Hey1* (−1.67-fold) and *Osr2* (−1.70-fold)] (Table S2). Network analysis placed *Lhx1* upstream of *Hnf1a* and *Hnf4a* as well as many components of the Notch pathway, such as *Hes5* and *Dll1* (Fig. 11A,B). Section ISH at E16.5 and P0 confirmed significant downregulation of *Hnf1a* and *Hnf4a* in HDAC1/2 mutant nascent tubules but preservation of *Hnf1b* (Fig. 11C). Similarly, the Notch ligands *Dll1* and *Jag1*, and several components of the Notch signaling pathway, including *Lfng*, *Osr2*, *Hes1* and *Hes5* were repressed (Fig. 11D). Consistent with downregulation of

Development (2018) 145, dev153619. doi:10.1242/dev.153619

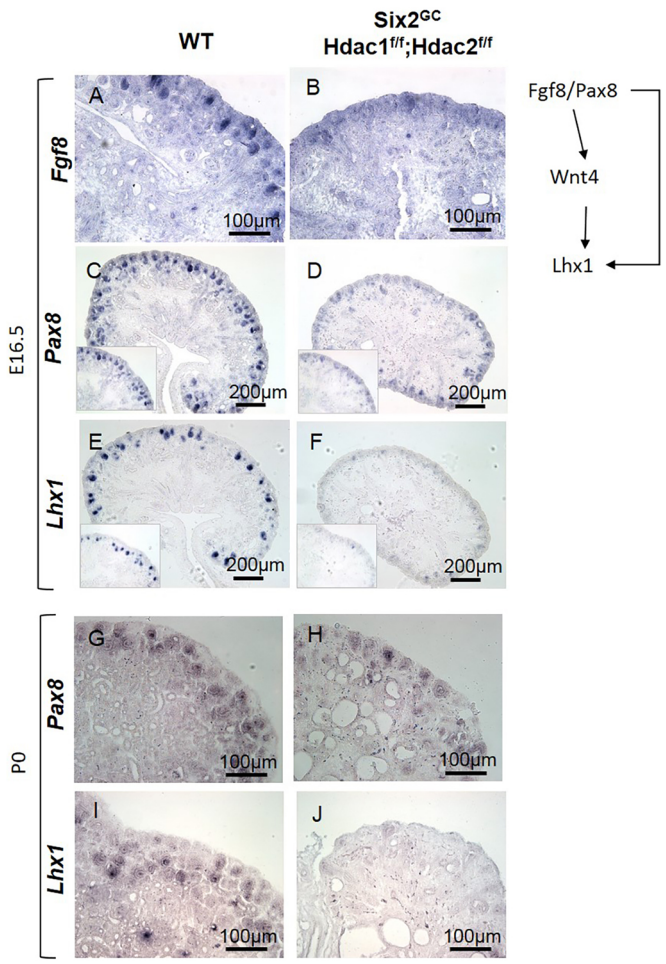

Fig. 9. HDAC1/2 deletion disrupts the RV regulatory network. (A-J) Section ISH at E16.5 (A-F) and P0 (G-J), showing early and persistent repression of *Pax8*, *Fgf8* and *Lhx1*, three major factors required for differentiation of RVs to nascent nephrons, in HDAC1/2 mutant kidneys.

the *Lhx1*/*Hnf1a*/*Hnf4a* network, there was a significant decrease in expression of the epithelial differentiation genes, such as *Nphs2*, *Slc34a1*, *Slc22a6*, *Slc37a4*, *CA4* (*Car4*) and *Cdh6*. In addition to the genes validated by section immunofluorescence (IF), ISH and reverse transcription (RT)-qPCR, NanoString gene expression analysis confirmed nine out of 11 randomly selected genes (Table S2). Unlike the differences in differentiation gene expression, which were readily identified using microarray analysis of whole kidney RNA, only a few progenitor genes were detected [e.g. decreased expression of *Cited1* (−6.35-fold) and *c-Myc* (*Myc*) (−1.59-fold)]. Section ISH and section IF readily detected changes in expression of these genes (e.g. *Pax2*, *Six2*, *Sall1* and *Osr1*) (Figs 4 and 5).

DISCUSSION

The present study provides comprehensive insights into the role of HDAC1 and HDAC2 in nephrogenesis. NPC-specific deletion of *Hdac1/2* genes caused downregulation of key progenitor genes, including *Six2*, *Pax2*, *Sall1*, *Wt1*, *Osr1*, *c-Myc* and *Cited1*, and premature depletion of NPCs. Our biochemical and ChIP analyses revealed that HDAC1/2 interact with Six2, Osr1 and Sall1, a network of transcriptional regulators that maintain the balance of NPC proliferation and differentiation, and that *Six2* is a potential *in vivo* target of HDAC1/2. Previous studies demonstrated that Sall1 is upstream of Six2, and Sall1 and Six2 are required for gene expression and cell renewal in the CM (Basta et al., 2014; Kanda et al., 2014). *Six2* or *Sall1* deletion results in depletion of nephron progenitors (Basta et al., 2014; Kanda et al., 2014; Kobayashi et al., 2008). Also, conditional deletion of the NuRD-specific component *Mi2b* (*Chd4*) in NPCs led to depletion of the CM (Denner and Rauchman, 2013). *Sall1* and *Mi2b* exhibit a strong genetic interaction in the developing kidney, supporting a cooperative role for Sall1 and NuRD in maintaining NPCs (Denner and Rauchman, 2013). Because HDAC1 and HDAC2 are key components of the NuRD complex, our data support a model in which the Sall1/Six2/HDAC complex is recruited to the *Six2* enhancer to maintain high Six2 expression in the NPCs.

Osr1 acts downstream of and interacts synergistically with Six2 and Groucho family transcriptional co-repressors to maintain the NPC pool via repression of Wnt4-directed nephrogenic differentiation (Xu et al., 2014). In *Osr1* mutant kidneys, *Wnt4* is ectopically activated throughout the CM, which undergoes premature mesenchyme-to-epithelium transition (Xu et al., 2014). Although our results showed protein-protein interactions between Osr1 and HDAC1/2, and Osr1 and HDAC1/2 target the *Wnt4* enhancer region, we did not observe ectopic Wnt4 activation or RV formation in the CM. We surmise that Osr1/Groucho interactions compensate for the loss of HDAC1/2 in NPC, thus preventing ectopic *Wnt4* activation in the CM.

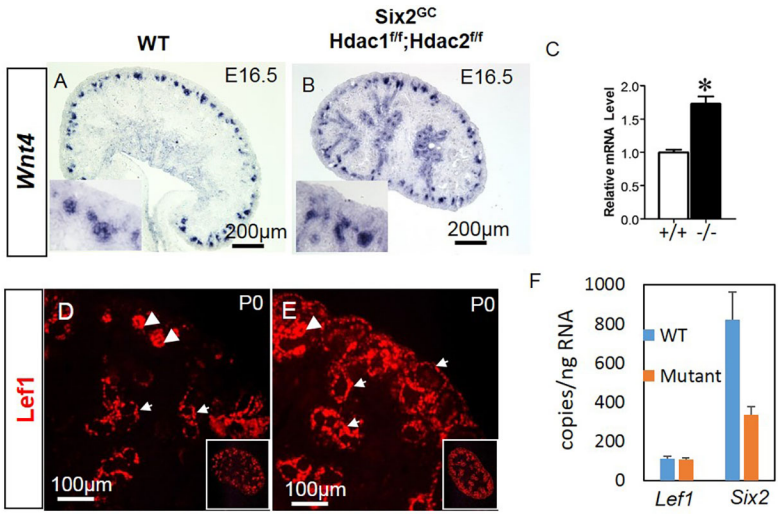

Fig. 10. Arrested RV differentiation in HDAC1/2 mutant kidneys is Wnt4 independent. (A-C) Section ISH shows maintained expression of *Wnt4* in the mutant RVs at E16.5 (A,B) and enhanced total kidney *Wnt4* by RT-qPCR (C). **P*<0.05; *n*=4. (D,E) Section IF at P0 using antibodies against Lef1. In WT kidneys, Lef1 is expressed in RVs (arrowheads) and in a thin layer of stromal cells surrounding the UB branches. In HDAC1/2 mutant kidneys, Lef1 is ectopically expressed in the stroma (small arrows) and is upregulated in the peri-UB stroma. (F) FACS-isolated NPCs from WT (*n*+3) and HDAC1/2 mutant (*n*=2) were cultured in differentiation medium (with 3.5 μM CHIR) for 48 h. ddPCR showed that the *Lef1* RNA copy number of mutant NPCs is not significantly different from that of WT cells, whereas the *Six2* RNA copy number of mutant NPC is significantly lower than that of WT cells. Data are mean±s.e.m.

Development (2018) 145, dev153619. doi:10.1242/dev.153619

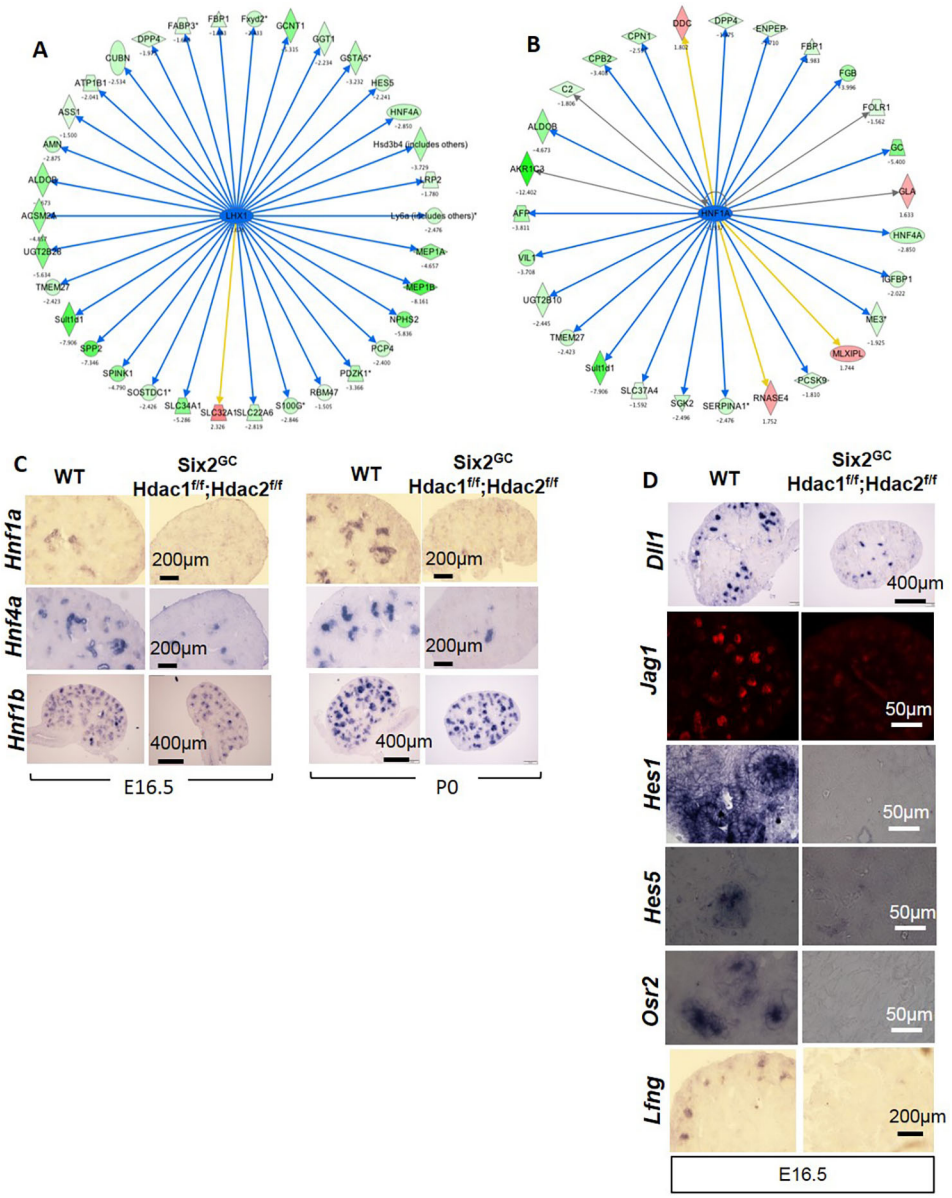

Fig. 11. Downregulation of the Notch/HNF network in *Six2*^{GC};*Hdac*$^{flox/flox}$, *Hdac2*$^{flox/flox}$ kidneys. (A,B) Network analysis of differentially expressed genes shows altered (mostly decreased) genes that are downstream of *Lhx1* and *Hnf1a*/*4a*. Green, downregulated; red, upregulated. Numbers indicate fold change versus WT. (C,D) Section ISH showing downregulation of *Hhf1a*/*4a* (but not *Hnf1b*) and Notch genes in HDAC1/2 mutant kidneys. HNF1A/HNF4A control expression of a network of genes involved in metabolic and transport functions in the proximal tubules. Decreased HNF1A-mediated gene expression is likely secondary to downregulation of Notch signaling, which controls segmental tubular differentiation.

In line with NPC depletion of HDAC1/2 mutant kidneys and the known pro-proliferative functions of HDAC1/2, we found that HDAC1/2 are crucial for NPC growth. Surprisingly, HDAC1/2 are not required to protect against p53-mediated apoptosis in NPCs. These findings are in sharp contrast to the effects of *Hdac1/2* deletion in the UB epithelium, where p53 hyperacetylation was accompanied by increased UB cell apoptosis, and concomitant deletion of p53 partially rescued the defect in UB branching morphogenesis (Chen et al., 2015). Here, we show that accumulation of acetylated p53 in NPCs does not induce aberrant apoptosis and concomitant deletion of p53 fails to rescue the renal dysgenesis in HDAC1/2 mutants. Collectively, these findings point to the differential and context-dependent functions of HDAC1/2 in NPCs versus UB epithelium, which warrants further studies.

In addition to premature NPC depletion, deletion of *Hdac1/2* blocks nephrogenesis at RV stage. This effect appears to be mediated via downregulation of the Lhx1/HNF/Notch network. *Wnt4* and *Lhx1* are both expressed in the RV, and *Lhx1* is genetically downstream of *Wnt4* (Kispert et al., 1998; Kobayashi et al., 2005; Stark et al., 1994). It is believed that the Wnt4-dependent transcriptional program leading to *Lhx1* activation serves to differentiate the pre-tubular aggregate into the epithelialized RVs (Halt and Vainio, 2014). Here, we show that deletion of *Hdac1/2* in NPCs abrogates *Lhx1* expression in RVs. Although downregulation of *Fgf8* and *Pax8* in HDAC1/2 mutant RVs can mediate *Lhx1* repression, it is conceivable that *Lhx1* is directly regulated by HDAC1/2 and is worthy of future study.

Aberrant Wnt4 expression in RVs in the face of downregulated expression of Pax8/Fgf8/Pax2/WT1 (upstream activators of Wnt4) was a surprising finding in this study. This suggests the presence of noncanonical upstream regulators of *Wnt4* gene expression within the RV that are unmasked by the loss of HDAC1/2. The nature of these factors remains to be determined. Our data also indicate that Wnt9b expression in the UB branches is not affected by the loss of HDAC1/2 in NPCs. Thus, the cause for maintained/enhanced Wnt4 expression in mutant HDAC1/2 RVs remains to be determined. Because our findings suggested that HDAC1/2 perform RV-specific functions, we attempted to delete *Hdac1/2* genes specifically from

Development (2018) 145, dev153619. doi:10.1242/dev.153619

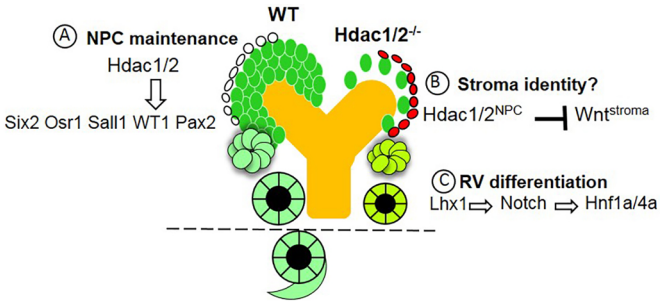

Fig. 12. Working model for the actions of HDAC1/2 in the regulation of nephrogenesis. HDAC1/2 perform sequential functions during NPC and epithelial differentiation. (A) In the NPCs, HDAC1/2 are recruited (possibly by Six2/Sall1) to Six2 enhancer where they serve a positive regulatory role. HDAC1/2 also interact with Osr1 in the maintenance of NPCs. (B) HDAC1/2 in the NPCs and/or nascent nephrons restrain Wnt protein activity in adjacent stroma via unknown mechanisms. (C) HDAC1/2 directly or indirectly regulate the RV transcriptional network (Pax8/Fgf8/Lhx1) upstream of the Notch/HNF1A/HNF4A pathway. The dotted line indicates that NPC-specific deletion of *HDAC1/2* arrests nephrogenesis at the RV stage.

the RVs using Wnt4-Cre/GFP mice. Although GFP was expressed appropriately in the RVs, we were unable to eliminate HDAC1/2 proteins, presumably as a result of the long half of the proteins, as each generation of nascent RVs express abundant HDAC1/2 prior to the deletion occurring. Genome-wide analysis of HDAC1/2-bound genes in NPCs versus RVs will be necessary to illuminate the direct versus indirect pathways regulated by HDACs during nephrogenesis.

In addition to Lhx1, many components of the Notch signaling pathway are downregulated in HDAC1/2 mutant kidneys. Notch signaling plays a key role in segmentation of the nephron and in the progression of pretubular aggregates/RVs towards comma- and S-shaped bodies during nephrogenesis (Kopan et al., 2007). γ-Secretase releases the Notch intramembrane domain (NICD), which, along with RBPJ, mediates the transcriptional effects of Notch proteins. Knockout of PSEN proteins (essential component of γ-secretase) or treatment with γ-secretase inhibitors allows formation of pretubular aggregates/RVs but not comma- and S-shaped bodies (Cheng et al., 2003; Wang et al., 2003). In addition, Notch2-deficient RVs initiate the segmentation process but fail to establish the proximal fate (Cheng et al., 2007), and more recently, the distal fate as well (Chung et al., 2016). Inactivation of *Lhx1* causes loss of Dll1, and Dll1 hypomorphic mice have a severe reduction in nephron numbers and the loss of proximal segments (Kobayashi et al., 2005). Following loss of Lhx1, at least three other Notch signaling components, Jag1, Hes5 and musashi homolg2 (Msi2), are repressed (Kobayashi et al., 2005). Our immunostaining and ISH results revealed that the expression level of Notch ligands (Dll1 and Jag1) and many Notch protein targets (Hes1, Hes5 and Osr2) that are important for nephrogenesis are also dramatically downregulated. Based on these results, we conclude that impaired Notch signaling contributes significantly to the developmental arrest at the RV stage and tubulogenesis failure of HDAC1/2 mutant kidneys.

In summary, the present study demonstrates that HDAC1/2 perform redundant, sequential and essential roles in the balance of NPC self-renewal and differentiation as well as in progression of nephrogenesis (Fig. 12). In wild-type NPCs, HDAC1/2, working in concert with Six2, Sall1 and Osr1, maintain expression of progenitor genes cell autonomously favoring the expansion of the multipotent nephron progenitor population. Additionally, HDAC1/2, presumably working together with other transcriptional regulators, are required to maintain the Pax8/Fgf8/Lhx1/HNF/Notch transcriptional regulatory network required for RV differentiation to nascent nephrons. It remains to be determined whether the effects of HDAC1/2 on NPC and RV transcriptional networks are mediated via locus-specific control/binding or more generalized effects on the epigenome. Future studies uncovering HDAC1/2 target genes in NPCs and RVs are clearly warranted to further understand the epigenetic regulation of nephrogenesis.

MATERIALS AND METHODS

Mice

Mice bearing conditional null alleles of *Hdac1*$^{flox/flox}$ and *Hdac2*$^{flox/flox}$ (Montgomery et al., 2007) were crossed to *Six2-CreEGFP* transgenic mice (*Six2-Cre*$^{tg/+}$) (Kobayashi et al., 2008) to delete the *Hdac1* and *Hdac2* genes, singly or in combination, specifically in the NPCs. *Wnt4GFPCre* BAC transgenic mice (Shan et al., 2010) were used to cross with *Hdac1*$^{flox/flox}$; *Hdac2*$^{flox/flox}$ mice to inactivate *Hdac1/2* in the RVs. *Trp53*$^{-/-}$ mice were obtained from the Jackson (JAX) Laboratory.

Histology and immunohistochemistry

Kidneys were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 µm. Histological analyses were performed by standard Hematoxylin and Eosin (H&E) staining. Section IF was performed as previously described (Chen et al., 2011, 2015). Antigen retrieval was accomplished by placing slides in 10 mM of boiling sodium citrate, pH 6.0, for 20 min. The following primary antibodies were used: anti-HDAC1 (1:100, 3601, BioVision), anti-HDAC2 (1:100, 3602, BioVision), anti-cytokeratin (1:200, C2562, Sigma-Aldrich), anti-Six2 (1:200, 11562-1-AP, Proteintechgroup), anti-Pax2 (1:200, 616000, Invitrogen), anti-PCNA (clone PC10, 1:200; DAKO Corp.), anti-cleaved caspase 3 (1:100, 9661s, Cell Signaling Technology), anti-acH4 (1:100, 06-866, Millipore), anti-acH3K9 (1:100, ab4441, Abcam), anti-p53AcK386 (1:100, ab52172, Abcam), anti-Jag1 (H-114; 1:100, sc-8303, Santa Cruz Biotechnology), Lhx1 (1:100, 4F2-C, Developmental Studies Hybridoma Bank), anti-Lef1 (1:100, 2230s, Cell Signaling Technology), anti-GFP (1:100, ab13970, Abcam), anti-E-cadherin (1:100, 610181, BD Biosciences), anti-Sall1 (1:100, ab31526, Abcam), anti-WT1 (1:100, ab15247, Abcam) and anti-laminin (1:100, L9393, Sigma-Aldrich). In negative controls, the primary antibody was omitted or replaced by nonimmune serum. For IF, the secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-rabbit (1:2000, Invitrogen) and anti-mouse fluorescein isothiocyanate (FITC) (1:200, Sigma-Aldrich). In addition, FITC-conjugated *Lotus tetragonolobus* lectin agglutinin (1:100, Vector Laboratories) was used to label the apical brush border of proximal tubules. Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) (1:500, D1306, Invitrogen). The immunofluorescent images were captured using a 3D or deconvolution microscope (Leica DMRXA2).

Section ISH

ISH was performed using digoxigenin-labeled antisense probes on kidney tissue fixed with 4% paraformaldehyde (PFA) as previously described (Chen et al., 2011). For section ISH, the kidney tissues were collected in DEPC-treated PBS, fixed in 4% PFA in diethyl pyrocarbonate (DEPC)-treated PBS overnight at 4°C, dehydrated in a series of alcohol, cleared in xylene and embedded in paraffin wax. Sections were cut to 10 µm thickness. After rehydration in 0.1% Tween in PBS, the samples were digested with proteinase K, and then refixed in 4% PFA, followed by 0.2% glutaraldehyde, followed by three washes in PBS. After a 3-h incubation in hybridization solution, the explants were hybridized with the digoxigenin-labeled antisense probes (~1 µg of probe/vial) overnight at 65°C. The next day, the samples were sequentially washed with hybridization solution, 2× saline sodium citrate (SSC), pH 4.5, 2× SSC, pH 7.0, 0.1% CHAPS, maleic acid buffer and PBS at room temperature. The slides were incubated with preblocked antibody (1:10,000, anti-Dig alkaline phosphatase, Roche Applied Science) at 4°C overnight. The following day,

Development (2018) 145, dev153619. doi:10.1242/dev.153619

after sequential washes of 0.1% bovine serum albumin 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 plasmids for *Dll1*, *Hes1*, *Hes5* probe preparation were gifts from Dr Ryoichiro Kageyama (Kita et al., 2007). The experimental and control samples were put in the same reaction vessel to allow for proper comparison. All the experiments, including ISH, immunostaining and TUNEL were repeated at least three times.

TUNEL assay

Apoptosis was assessed using TUNEL and was carried out using an *in situ* apoptosis detection kit (Trevigen) according to the manufacturer's guidelines. Four-micrometer paraffin sections were fixed in methanol-free PFA before and after proteinase K treatment at 20 µg/ml for 8-10 min at room temperature. The sections were incubated with the nucleotide mixture (which included fluorescein-tagged dUTP) and rTdT enzyme for 1 h at 37°C. The slides were mounted using Vectashield with DAPI (Vector Laboratories). The images were captured using a deconvolution fluorescent microscope.

Cell culture and transient transfection

HEK 293T cells were obtained from the laboratory of Dr Hua Lu (Tulane University, New Orleans, LA, USA, and Johns Hopkins University Cell Center, Baltimore, MD, USA). Cells were grown in high-glucose Dulbecco's modified Eagle medium (DMEM) with stable glutamine supplemented with 10% fetal bovine serum (FBS) and 10 mg/ml antibiotics (penicillin and streptomycin). All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells seeded on the plate overnight were transfected with plasmids as indicated in figure legends using Lipofectamine LTX with Plus Reagent following the manufacturer's protocol (Thermo Fisher Scientific). Cells were harvested at 48-72 h post-transfection. The plasmid Flag-Sall1 was a gift from Dr Ryuichi Nishinakamura (Kanda et al., 2014), Flag-Six2 and Myc-Six2 were gifts from Dr Joo-Seop Park (Park et al., 2012), and Flag-Osr1 was a gift from Dr Rulan Jian (Xu et al., 2014).

Immunoprecipitation and western blotting

Immunoprecipitation (IP) was conducted using antibodies as indicated in the figure legends. Briefly, ∼500-1000 µg of protein was incubated with the indicated antibody at 4°C for 4 h or overnight. Protein A or G beads (Santa Cruz Biotechnology) were then added, and the mixture was incubated at 4°C for an additional 1-2 h. Beads were washed at least three times with lysis buffer. Bound proteins, as well as the whole-cell extracted proteins, were detected by western blotting. The following primary antibodies were used: anti-HDAC1 (rabbit polyclonal, 1:1000, BioVision) and anti-HDAC2 (rabbit polyclonal, 1:1000, BioVision), anti-Flag (1:1000, Sigma-Aldrich), anti-Myc (1:1000, Sigma-Aldrich) and mouse anti-β-actin (1:5000, Sigma-Aldrich).

Isolation of NPCs

$Six2^{+}$/$Cited1^{+}$ NPCs were isolated from E16.5 kidneys by magnetic-activated cell sorting (Brown et al., 2015). Briefly, after dissecting kidneys and removing the capsule, the kidneys were digested in collagenase A/pancreatin enzyme digest solution at 37°C for 15 min. After digestion, FBS was added to the tube containing kidneys to stop the enzyme reaction. The cell suspension was transferred to new microfuge tubes and cells were collected by centrifugation at 300 ***g*** for 5 min. Subsequently, the cells were re-suspended and filtered through a 30-µm pre-separation filter. Magnetic depletion was carried out through the addition of anti-CD105-PE, anti-CD140-PE, anti-Ter119-PE and anti-CD326-PE. Finally, the NPCs ($Cited1^{+}$ NPCs) were collected as the negative fractions.

RNA isolation and ddPCR

E16.5 NPCs were isolated, plated and expanded on Matrigel-coated plates in a monolayer in keratinocyte serum-free medium (Gibco) supplemented with 50 ng/ml FGF2 (R&D Systems) as described (Brown et al., 2011, 2013) for 48 h. Total RNA was isolated using an RNA isolation kit (Qiagen). RNA concentration was quantified using Nanodrop 2000 (Thermo Fisher Scientific). ddPCR was performed on a Bio-Rad ddPCR system to determine *Lef1* and *Six2* gene expression. All reagents for the One-Step RT-ddPCR system were purchased from Bio-Rad to generate complementary DNA (cDNA) and quantify gene expression. Droplets were analyzed on the QX200 droplet reader and target cDNA concentration was determined using QuantaSoft analysis software (Bio-Rad).

ChIP

ChIP experiments were performed using an EZ ChIP chromatin immunoprecipitation kit (17-371, Millipore) according to the manufacturer's protocol. Immunoprecipitation was performed with ChIP-grade antibodies to HDAC1 (ab7028, Abcam), HDAC2 (ab7029, Abcam), Six2 (11562-1-AP, ProteinTech). Rabbit IgG (ab46540, Abcam) was used as a control antibody. 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) were analyzed by PCR. The primer sequences used for PCR were:

Six2Enh Forward: 5′-ACCGGATGGAAAGGCTTTAT-3′
Six2Enh Reverse: 5′-GGGCTGTTCCAGCTACAGAG-3′

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 a Fluorescent Direct Label Kit (Agilent) and 1.0 mM cyanine 3′- or 5′-labeled dCTP (PerkinElmer). Hybridization was performed using an 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 GSE84305.

Data analysis

MultiExperiment Viewer v4.9 software was used to generate lists of genes differentially expressed between WT and HDAC1/2 mutant kidneys, using $P<0.05$ and a minimum 1.5-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, 2015). Additional analysis of the microarray data was accomplished using IPA software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: H.L., S.S.E.-D.; Methodology: S.C., Y.L., C.-H.C., J.L., Z.S.; Validation: Y.L.; Formal analysis: H.L.; Investigation: H.L., S.C., X.Y., C.-H.C., J.L., Z.S.; Resources: S.S.E.-D.; Data curation: H.L., S.C., X.Y.; Writing - original draft: H.L., S.S.E.-D.; Writing - review & editing: S.C., Z.S., S.S.E.-D.; Supervision: S.S.E.-D.; Project administration: H.L., S.S.E.-D.; Funding acquisition: H.L., S.S.E.-D.

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Data availability

Microarray analysis data are available at GEO under accession number GSE84305.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.153619.supplemental

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