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Dot1l deficiency leads to increased intercalated cells and upregulation of V-ATPase B1 in mice

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ABSTRACT

The collecting duct in the mammalian kidney consists of principal cells (PCs) and intercalated cells (ICs), which regulate electrolyte/fluid and acid/base balance, respectively. The epigenetic regulators of PC and IC differentiation remain obscure. We previously used Aqp2 and V-ATPase B1B2 to label PCs and ICs, respectively. We found that mice with histone H3 K79 methyltransferase *Dot1l* disrupted in Aqp2-expressing cells (*Dot1l*^{AC}) vs. *Dot1l*$^{f/f}$ possessed ~20% more ICs coupled with a similar decrease in PCs. Here, we performed multiple double immunofluorescence staining using various PC and IC markers and confirmed that this finding. Both α-IC and β-IC populations were significantly expanded in *Dot1l*^{AC} vs. *Dot1l*$^{f/f}$. These changes are associated with significantly upregulated V-ATPase B1 and B2, but not Aqp2, AE1, and Pendrin. Chromatin immunoprecipitation assay unveiled a significant reduction of Dot1l and H3K79 di-methylation bound at the *Atp6v1b1* 5′ flanking region. Overexpression of Dot1a significantly downregulated a stably-transfected luciferase reporter driven by the *Atp6v1b1* promoter in IMCD3 cells. This downregulation was impaired, but not completely abolished when a methyltransferase-dead mutant was overexpressed. Taken together, our data suggest that Dot1l is a new epigenetic regulator of PC and IC differentiation and *Atp6v1b1* is a new transcriptional target of Dot1l.

1. Introduction

A critical role of the renal collecting duct (CD) in the mammalian kidney is the regulation of electrolyte, fluid and acid–base homeostasis. CD consists of principal cells (PCs) and intercalated cells (ICs) [1], which are structurally and functionally distinct. PCs reabsorb Na^+ and water. Na^+ enters into PCs from the urine through the apical epithelial Na^+ channel (ENaC) and exits into blood through the basolateral Na^+–K^+-ATPase. A similar process occurs for water reabsorption mediated by the coordinated actions of water channels Aqp2 at the apical side and Aqp3 and Aqp4 at the basolateral side. These Na^+ and water channels thus serves as the molecular markers of PCs. ICs are responsible for the acid–base balance and express carbonic anhydrase type II (CAII) and the proton-pumping V-ATPase. The latter contains 13 or more subunits such as A, B1 and B2. CAII and V-ATPase subunits are also used to identify ICs in the kidney. ICs are further divided into α-ICs and β-ICs. α-ICs are involved in acid secretion and marked by expression of AE1, the kidney variant of the band $3Cl^-/HCO_3^-$ exchanger. β-ICs secrete bicarbonate and are characterized by expression of the sodium-independent chloride/iodide transporter Pendrin [2].

The relative abundance of the three cell types in kidney can be affected by metabolism and the genes involved in the differentiation. For example, metabolic acidosis reduced β-ICs and increased α-ICs without changing the total IC population [3]. ICs as evaluated by detectable AE1 and H^+-ATPase expression are severely depleted and replaced by PCs in CAII knockout mice. Hensin/DMBT1 encodes an extracellular matrix protein. Mice deficient in Hensin/DMBT1 contained no typical α-ICs [4]. Disruption of the forkhead transcription factor Foxi1 led to generation of a single cell type ($Aqp2^+$ $CAII^+$) that have no detectable expression of other IC markers including V-ATPase B1, Pendrin, and AE1 [5].

Disruptor of telomeric silencing (*Dot1*) was originally identified in yeast through a screen aimed to identify genes affecting telomeric silencing [6]. Biochemical analyses led to identification of *Dot1* and its mammalian homologs (*Dot1l*) as the genes encoding a

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family of methyltransferases specific for histone H3 K79 [7–9]. Dot1l is involved in a variety of biological processes including development, erythropoiesis, differentiation, proliferation, and leukemogenesis [10–12], most likely by regulating transcription of target genes. General knockout of *Dot1l* results in embryonic lethality in mice [13].

Mouse *Dot1l* generates 5 alternative splicing variants that we named as Dot1a–e [9]. Among these variants, Dot1a and Dot1b are the only ones whose open reading frames have been characterized. Dot1a has been shown to promote H3 K79 methylation and represses the transcription of the epithelial Na^+ channel αENaC and several other aldosterone-regulated genes in 293T cells, mouse cortical collecting duct M1 cells, mouse inner medulla collecting duct IMCD3 cells, and mouse kidney [14–16]. Consistently, Northern blotting and immunofluorescence staining analyses revealed strong expression of Dot1l mRNAs and H3 dimethyl K79 (m2K79) in mouse kidney [9,16]. Most recently, using *Aqp2Cre* PAC transgene driver, we generated $Dot1l^{AC}$ mice that have disrupted *Dot1l* function in Aqp2-lineage cells [17]. Deletion of *Dot1l* in these cells was validated by complete loss of mono-, di-, and tri-methylated H3 K79 without affecting methylation in all other residues examined. Since Dot1l is the sole enzyme mediating H3 K79 methylation in mice, we used loss of di-methylated H3 K79 (H3m2K79) as a marker to trace Aqp2 lineage. We found that both PCs and most of ICs are derivatives of a common ancestral population that expresses Aqp2. Moreover, $Dot1l^{AC}$ vs. $Dot1l^{f/f}$ mice have ~20% less of PCs and ~16% more ICs, as evaluated by double immunofluorescence staining using two specific antibodies. One of them recognizes Aqp2 and the other labels both of V-ATPase subunits B1 and B2 [17]. Consistently, $Dot1l^{AC}$ vs. $Dot1l^{f/f}$ mice exhibited relatively hypoosmotic polyuria on a normal chow diet or after 24-h water deprivation [17,18].

In this report, we further demonstrated that $Dot1l^{AC}$ vs. $Dot1l^{f/f}$ mice had decreased PCs and increased ICs including both α-ICs and β-ICs, reduced V-ATPase B1 mRNA levels, and diminished Dot1l and H3 K79 di-methylation bound at the *Atp6v1b1* 5′ flanking region. We also show that Dot1a inhibited *Atp6v1b1* promoter activity, which is largely dependent on its methyltransferase activity, in IMCD3 cells. This study highlights the function of *Dot1l* in regulating PC and IC differentiation and addition of *Atp6v1b1* as a new member in the growing list of Dot1l target for transcriptional regulation.

2. Materials and methods

2.1. Reagents

The primary antibodies used are chicken anti-Aqp2 LC54 (a gift from James B. Wade, University of Maryland School, Baltimore, MD), chicken anti-V-ATPase B1 [19], rabbit anti-AE1 (Alpha Diagnostic, 396768A3), anti-H3 di-methyl K79 (abcom), and five antibodies from Santa Cruz: goat anti-Aqp2 (sc-9882), goat anti-Aqp3 (sc-9885), goat anti-Pendrin (sc-16894), and rabbit anti-Aqp2 (sc-28629). The secondary antibodies are Dylight 594-AffiniPure goat anti-chicken IgG (Jackson ImmunoResearch LABORATORIE) and from Invitrogen Alexa Fluor 488-conjugated goat anti-mouse IgG (774904), Alexa Fluor 488-conjugated donkey anti-goat IgG (51475A), Alexa Fluor 594-conjugated donkey anti-mouse IgG (796011), and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (A10042). Constructs expressing WT or methyltransferase-dead mutant Dot1a were described [9]. The *Atp6v1b1* 5′-flanking region (0.84 kb; −842 to −1, relative to the transcription start site) was amplified by PCR with the genomic DNA isolated from a $Dot1l^{f/f}$ mouse as the template. The fragment was inserted into pGL3Zeocin at the MluI-XhoI sites to generate pGL3Zeocin-0.84mVB1 for stable transfection.

Mouse models. $Dot1l^{f/f}$, *Aqp2Cre* and $Dot1l^{AC}$ mice have been previously described [10,20]. Littermates from the cross between $Dot1l^{f/f}$ and $Dot1l^{AC}$ mice were used for all of the current studies. Following weaning, mice were maintained with a standard pellet chow and used at the age of 2–5 months for immunofluorescence, real-time RT-qPCR, and chromatin immunoprecipitation (ChIP) analyses.

All animal experiments were approved by the University of Texas Health Science Center at Houston Animal Welfare Committee and carried out according to NIH Guides for the Care and Use of Laboratory Animals.

2.2. Immunofluorescence studies

Immunofluorescence (IF) staining was conducted and analyzed as we reported before [21,22], with following modifications. IF images were examined and scored for the staining of each primary antibody with the aid of Adobe Photoshop CS4. To gauge the relative percentages of the 4 cell types in each double IF, we examined 3–4 mice/genotype in the related double IF experiments. Five to ten fields were examined from the whole kidney in each mouse. About 900 cells from > 100 CNT/CD tubules in the cortex, outer medulla and inner medulla were counted and categorized on the basis of the staining of each primary antibody used. CNT/CD structures were defined by possessing at least one PC or IC. Any structures lacking a PC or IC were excluded from cell counting. In the IF experiments using anti-Aqp2 or anti-Aqp3 antibodies, CNT/CD segments were recognized by the existence of at least one $Aqp2^+$ or $Aqp3^+$ cells, respectively. It is possible that the populations of CNT/CD structures defined by the presence of at least one $Aqp2^+$ or $Aqp3^+$ cells are not necessarily identical, leading to fluctuations in the relative percentages of PCs and ICs in different IF experiments of the same genotype.

2.3. Genotyping, luciferase assay, real-time RT-qPCR, and ChIP assay

These assays were performed according to our published protocols [14,16,23,24]. For luciferase assay, pGL3Zeocin-0.84mVB1 was transfected into IMCD3 cells. Cells were selected with Zeocin (800 μg/ml) for 3 weeks, with medium changes every 2–3 days. Survival cells were pooled to establish a stable cell line for transient transfection of Dot1a constructs. All primer sequences are available upon request.

2.4. Statistical analysis

Quantitative data are shown as mean ± SEM. Statistical significance was evaluated via Student *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Double immunofluorescence analyses revealed significantly reduced PCs and increased ICs in $Dot1l^{AC}$ vs. $Dot1l^{f/f}$ mice

Previous double immunofluorescence (IF) analyses with a rabbit anti-V-ATPase subunits B1 and B2 and a goat anti-Aqp2 revealed that $Dot1l^{AC}$ vs. $Dot1l^{f/f}$ mice have increased ICs to the detriment of PCs. To further solidify this finding, we first conducted double IF by replacing the anti-V-ATPase subunits B1 and B2 with an antibody specific for V-ATPase B1 (B1) only. This is because B1 and B2 display differential expression patterns, with B1 expression being restricted to ICs and B2 ubiquitous [25,26].

The staining of the two antibodies differentiated the cells in the