p63+ ureteric bud tip cells are progenitors of intercalated cells

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Introduction

The developing kidney is derived from the caudal intermediate mesoderm by iterative mesenchymal-epithelial interactions between the metanephric mesenchyme and ureteric bud (UB) cell lineages. The collecting ducts, which are derivatives of the UB, play a critical role in the maintenance of salt, water, and acid-base homeostasis. These physiological functions are carried out by highly specialized cell types: the principal cells (PC) and intercalated cells (IC). Embryologically, PC and IC are believed to be derived from bipotent UB precursors, which are themselves derived from stem/progenitor cells located at the UB tips (1). Insults that disrupt the ureteric bud tip cell’s (UBTC’s) capacity for cell renewal and/or terminal differentiation have the potential to cause structural defects and dysregulation of blood pressure, electrolyte and water balance, and acid-base homeostasis (2, 3). Designing strategies to hasten collecting duct regeneration following injury and rebuilding a functional collecting duct from pluripotent cells will benefit from a better understanding of UBTC fate determinants.

While much is known about the cellular and molecular mechanisms governing UB growth and branching, relatively little is known about the dynamic regulation of UBTC differentiation. Careful lineage analysis has established that UBTC are the progenitors of the entire collecting duct epithelium (4–6). In response to glial-derived neurotrophic factor released from the surrounding mesenchyme, Ret+ UBTC undergo proliferation, changes in cell shape, and cell movements (collectively, a process called mitosis-associated cell dispersal) to mediate UB growth and branching and, ultimately, form the renal collecting system (5). There is limited knowledge of the UBTC transcriptome at the earliest stages of mouse and rat kidney development (7). Current models posit that UBTC give rise to bipotent UB precursors capable of differentiating to PC or IC and that Notch and Foxi1 signaling dictate the decisions for fate commitment and/or are required for fate maintenance (2, 8, 9). However, a number of fundamental questions remain unanswered. For example, can UBTC differentiate directly to PC or IC without passing through the bipotent stage? Where does the commitment step take place (UB tip vs. trunk)? And, what are the transcription factors that specify the cell type fates?
p63 (also known as TP63) is a member of the p53 gene family of transcription factors (which comprises p53, p63, and p73). The p63 gene is transcribed into full-length (TAp63) and N-terminus–truncated (ΔNp63) transcripts via differential promoter utilization. Differential splicing of p63 also generates multiple C-terminus isoforms, the exact functions of which are largely unknown (10–14). p63 is expressed in the basal layer of stratified epithelia such as the skin, lung, and limbic epithelium. Genetic studies have shown that p63 is absolutely required for epithelial tissue morphogenesis (15–19), as mice lacking p63 isoforms die in the perinatal period due to severe skin disease and dehydration. Emerging data also implicate p63 in the regeneration program of the alveolar epithelium following injury (20). As stated earlier, traditionally, p63 expression is observed in basal epithelial stem cells of stratified epithelia. Here, we show for the first time to our knowledge that p63 is expressed in the UB tip domain, which consists of a simple progenitor epithelium. p63 expression in the UB tip is transient and is silenced by the end of nephrogenesis. Moreover, a subset of UBTC expressing the ΔNp63 isoform gives rise to cortical IC. Finally, gene targeting in mice directly implicates ΔNp63 in IC differentiation. Thus, in sharp contrast to existing models of collecting duct differentiation, our findings suggest that UB tip progenitors are prepatterned and fate restricted.

**Results**

*p63 transiently marks UBTC in mouse and human kidneys.* Although p63 expression in the urothelium is well described, to our knowledge, its expression in the nephron epithelium has not been reported previously. To characterize the spatiotemporal expression of p63 during kidney development, we immunostained sections of embryonic (E12.0–E17.5) and postnatal kidneys (P0, P3, P5, P10, and P60) with monoclonal p63 antibodies (clones 4A4 and SFI-6), which recognize both ΔNp63 and TAp63 isoforms. At E12.0, the UB had branched 2–3 times; both UB and its branches were p63 negative (Figure 1A). p63 expression in the UB epithelium was first detectable at E13.5, but at this stage, p63 was not detectable in the UB branches or tips (Figure 1B). E15.5 marked the onset of p63 expression in UBTC (Figure 1, C and D). At E17.5, p63 was abundantly expressed in UBTC, and it remained so until it was permanently silenced by P5 (Figure 1, E–I). After P5, p63 immunoreactivity was limited to the pelvic urothelium (Figure 1J). Collectively, these findings demonstrate that p63 is a marker of UBTC and is expressed only transiently during kidney development.

The c-Ret tyrosine kinase receptor marks the UB tip after E12.5, and Ret signaling is absolutely required for UB outgrowth and branching (21). We asked if p63 expression overlaps with that of Ret. For this purpose, we used Ret-EGFP knockin mice (22). In these mice, EGFP expression is regulated by the Ret endogenous regulatory elements. We observed that p63 labels a subset of GFP+ UBTC (Figure 2). The distribution of p63+ cells within the UB tip domain was not spatially associated with branching points.

We next asked if p63 expression in the UB tip, observed in the mouse kidney, is conserved in human kidneys. Immunostaining of a fetal human kidney at 12 weeks after conception revealed that p63 was expressed in the main UB branches (Figure 3, A–D) and UB tips (Figure 3E). Thus, expression of p63 in UBTC is evolutionarily conserved and, therefore, is likely to have functional importance.

ΔNp63+ UBTC give rise to cortical IC. Previous studies have shown that ΔNp63 labels keratinocyte, lung, and prostate stem cells and is required for their maintenance and differentiation (16, 19, 20, 23–25). We asked whether ΔNp63 serves a similar function in the UB progenitors. To this end, we first defined the spatial expression of ΔNp63 in the UB tip. Using ΔNp63 isoform–specific antibody (26), we found 1–2 cells per tip that stained positive for ΔNp63 (Figure 4, A–D). We also examined knockin ΔNp63+/Cre;R26RED2YFP or R26REMt/mG mice (which express GFP under the control of the endogenous ΔNp63 regulatory elements) (26). We clearly identified GFP+ UB tips/cells in whole mount kidneys (Figure 4E) and tissue sections (Figure 4F), respectively. Costaining of GFP with p63 and Pax2 revealed that ΔNp63-expressing cells constituted a subset of total p63+ tip cells, whereas the cap mesenchyme was devoid of p63 (Figure 4, G–I).

We next used mice carrying a knockin ΔNp63+/Cre allele (19) to trace the fate of ΔNp63+ UBTC (UBTCΔNp63+/Cre). We performed single- or double-label immunostaining in fetal and neonatal kidney sections from ΔNp63+/Cre;R26RED2YFP or R26REMt/mG mice. At E17.5–E18.5, we documented the presence of 1–3 EYFP+/p63+ cells per UB tip domain (Figure 5), indicating that we are likely detecting the earliest ΔNp63-expressing cells. As expected, the pelvic urothelium contained p63+/EYFP+ and daughter p63+/EYFP+ ΔNp63+/Cre cells (Figure 5A). By P1, labeled ΔNp63+/Cre cells were detectable not only in the UB tip, but also in the collecting ducts (Figure 6, A–D). While we found labeled ΔNp63+/Cre cells in the pelvic epithelium, we did not observe any labeled cells in the papillary collecting ducts, making it highly unlikely that labeled cells found in the cortical collecting ducts migrated up from the pelvic epithelium (Figure 6E).
In the adult kidney, although p63 protein was no longer detectable (silenced by P5), GFP+ ΔNp63lineage cells were clearly visible in the cortical collecting ducts and costained with the canonical IC marker, vATPaseB1, but not the PC marker, AQP2 (Figure 7, A and B). Previous studies have shown that cortical pendrin+ type B-IC give rise to AE1 + type A-IC (27, 28). Consistent with this finding, while some labeled ΔNp63lineage cells expressed luminal pendrin, others expressed basolateral AE1 (Figure 7, C and D). We did observe rare labeled ΔNp63lineage cells in the medulla, but they did not harbor IC markers (Figure 8).

In summary, our immunolocalization and cell fate studies unraveled a noncanonical differentiation pathway involving ΔNp63+ UBTC, which are fate restricted early, giving rise to cortical IC (Figure 7E).

ΔNp63 deletion disrupts IC differentiation. Germline ΔNp63+/− knockin mice survive the embryonic period but die postnatally of severe skin disease (26), precluding the ability to examine the adult kidney phenotype. However, histological and immunohistochemical analysis of late-gestation ΔNp63+/− mice revealed that heterozygous or homozygous deletion of ΔNp63 has no discernible effect on UB integrity or branching (Figure 9, A and B, and data not shown). The residual p63 immunoreactivity in ΔNp63+/− kidneys (Figure 9, C and D) likely reflects the TAp63 isoform, which also localizes to the UB tip (Saifudeen and Liu, unpublished observations). Importantly, although loss of ΔNp63 did not alter the integrity of GFP+ tip progenitors (Figure 9, C and D, insets), it caused a remarkable reduction (but not complete elimination) in abundance of cortical CAII+ IC (Figure 9, E and F). Thus, ΔNp63 is required for the maintenance (renewal, survival, or differentiation) of a subset of UB tip progenitors that are able to transition into IC. The precise mechanism(s) will need to be addressed in future studies.

Discussion
The present study demonstrates that the transcriptional regulator, p63, is a specific marker of Ret+ UBTC, which are the progenitors of all cell types of the mature collecting duct. Interestingly, expression of p63 in the UB tip is transient, beginning around E15 and ending at P5, thus coinciding temporally with the initiation of specification and terminal differentiation of the collecting duct. Fate tracing of UBTC expressing the ΔNp63 isoform revealed that UBTC+ΔNp63lineage give rise to cortical IC. Genetic deletion of ΔNp63 interferes with UBTC differentiation into cortical IC. Collectively, these findings have uncovered
a transcriptional regulator of UBTC differentiation and demonstrate that at least a subset of UBTC progenitors are sorted out early and differentiate into IC.

During kidney development, as the UB branches and elongates, the tip and trunk domains are patterned into progenitors and progeny, respectively. This tip-to-trunk program is reflected by differences in gene expression (7). While it is recognized that UBTC are the progenitors of all collecting duct cell types (29), the mechanisms that balance their renewal and differentiation are not well defined. Local Wnt–β-catenin signaling may prevent premature differentiation of UBTC, probably acting via Sox9 and Emx2 (30, 31). Extrinsic factors released from the cap mesenchyme, stroma, or neighboring epithelial elements may also impinge on UBTC differentiation. Regardless of the initial differentiation signal, current models posit that UBTC progenitors differentiate into bipotential UB precursors (1, 32), and that acquisition of Foxi1 activity in these precursors jump starts the IC differentiation program (32), as Foxi1−/− mice develop distal renal tubular acidosis due to lack of IC (33). In contrast, Notch signaling is necessary to initiate and maintain the PC fate and, possibly, to suppress Foxi1 signaling. This was illustrated in mice lacking mindbomb-1 (mib-1), an E3 ubiquitin ligase that mediates ligand processing in the Notch-releasing cell. Mib-1−/− deficient kidneys have a markedly reduced PC/IC ratio, although the overall PC and IC patterning in the collecting duct remained unaltered (34).

An untested hypothesis for UBTC differentiation is that a small subset of tip cells are sorted out early in the process for either PC or IC fate under the influence of a specific developmental regulator. Our findings lend support to this hypothesis. In the present work, we report that UBTCΔNp63 lineage consistently give rise to cortical IC (both B type and A type). Although occasional UBTCΔNp63 lineage labeled cells were found in the medullary collecting ducts, these cells lacked vB1-ATPase expression. Therefore, our findings support the idea that cortical type B IC give rise to type A IC, previously proposed by Al-Awqati (reviewed in ref. 2). The cellular precursor of medullary type A IC remains to be determined.

The present study provides several lines of evidence supporting the idea that ΔNp63 participates...
in programming UBTC-to-IC fate. (a) ΔNp63 is expressed at the right location (UB tip) and time (late gestation), which coincides with collecting duct cell functional specification. (b) ΔNp63 acts transiently, as it is completely silenced soon after birth. This finding suggests that ΔNp63 initiates the UBTC-to-IC program, but its presence is not essential to maintain the differentiation state; another factor such as Foxi1 may play such a role. Indeed, loss of Foxi1 is accompanied by accumulation of precursor cells expressing both PC and IC markers (33), confirming that Foxi1 is necessary for the later stages of IC specification. (c) Genetic deletion of ΔNp63 results in a reduction of the cortical IC mass. Unfortunately, ΔNp63–/– mice die shortly after birth, precluding the full characterization of the mature phenotype and physiological effect on the urinary acidification mechanism. Our attempts at sampling the amniotic fluid of late-gestation ΔNp63–/– mice failed due to the contamination of the fluid with debris from sloughed skin and epidermal tissues due to extreme fragility of the skin and the amniotic sac. Although this issue cannot be addressed at the present time, based on the findings in the present study, we predict that UB-specific deficiency of ΔNp63 will lead to permanent loss of

Figure 4. ΔNp63 is expressed in a subset of ureteric bud tip cells. (A–D) Section immunofluorescence of an E17.5 kidney using ΔNp63-specific antibody. Staining for cytokeratin (CK) identifies the ureteric bud (UB) branches. Arrows point to ΔNp63+ cells in B–D. RV, renal vesicle. (E) Whole mount GFP fluorescence in an E18.5 kidney from a ΔNp63 Cre reporter knockin mouse. Note the tip expression of GFP. (F) Section immunofluorescence using anti-GFP antibody in an E18.5 ΔNp63 Cre reporter kidney showing UB tip–specific expression of GFP. (G–I) Section immunofluorescence using anti-GFP, p63, and Pax2 antibodies in an E18.5 ΔNp63 Cre reporter kidney. p63 (red) labels the UB tip domain (UBT). Pax2 (gray) labels the UB tip (UBT) and surrounding cap mesenchyme (CM). GFP (green), representing ΔNp63, is expressed in a subset of p63+ ureteric bud tip cells (n = 2–5 animals/experiment). Original magnification, ×4 (E); ×10 (F); ×40 (A–D and G–I).

Figure 5. Fate tracing of UBTCΔNp63 in E17.5 ΔNp63 Cre;ROSA26EYFP mice. (A and B) Section immunohistochemistry. p63 (brown) strongly labels the ureteric bud tip cells (UBTC) and pelvic urothelium. EYFP (purple) labels 1–2 UBTC (white arrowheads) and patches of pelvic epithelial cells (black arrowheads). (C and D) Colocalization of p63 protein (purple) with EYFP (green) in UBTC (n = 5). Arrowheads point to p63+/EYFP+ cells. Original magnification, ×10 (A); ×20 (B); ×40 (C and D).
acid-secreting cells and renal tubular acidosis.

Although this study was not designed to address the mechanisms whereby ΔNp63 programs the UBTC-to-IC fate, several ideas come to mind. ΔNp63 may be required for the renewal or survival of a select subset of UBTC destined to give rise to IC; however, this hypothesis is countered by the integrit of GFP+ tip cells, which remains intact in knockin ΔNp63 gfp/gfp mice. Another possibility is that ΔNp63 acts upstream of Foxi1, a key transcriptional regulator of IC genes (32). A ChIP-seq screen of p63 target genes in neonatal kidney cortex did not reveal binding of p63 to the Foxi1 gene (Saifudeen and El-Dahr et al., unpublished observations), suggesting that Foxi1 is not a direct p63 target gene. We did, however, find binding of p63 to multiple genes in the Notch pathway, including Notch1, Notch2, Notch4, MAML3, Hes7, DLL1, Jag1,2 and RFNG. A previous study demonstrated binding of ΔNp63 to the Notch3 gene in epidermal cells (26). It is tempting to speculate that ΔNp63 promotes the IC fate indirectly by repressing the Notch pathway. It is also conceivable that ΔNp63 “bookmarks” IC-specific genes, facilitating binding and activation by Foxi1. In this regard, ΔNp63 has been shown to recruit chromatin remodelers to dynamic enhancers of epidermis-specific differentiation genes (35).

The coexpression of p63 in Ret+ tip progenitors prompts the question of whether p63 is a genomic target of Ret signaling. Review of public databases and the published UBTC transcriptome do not support this hypothesis. On the other hand, there is evidence that p63, jointly with Sox2, regulates the Ret target ETV4 gene (36). Since p63 expression is ini...
tiated later than Ret in the developing kidney, it is still possible that GDNF/Ret or other tyrosine kinases may be upstream of p63. Future studies using conditional Ret or ETV4 mutants are needed to address this question.

In summary, the present study suggests that UBTC progenitors are not homogenous; at least a subset is specified early on to differentiate into cortical IC under the influence of ΔNp63. Future studies are needed to address how p63 programs the fate of UBTC progenitors and whether these mechanisms can be exploited in regenerative medicine.

**Methods**

**Mice.** ΔNp63+/Cre knockin mice were generated in the Signoretti laboratory (ref. 19) and were used to drive recombinase activity in ΔNp63-expressing cells. Genotyping of these mice was performed as described in ref. 19. ΔNp63+/Cre mice were crossed with the B6.129 X 1-Gt(ROSA)26Sor m1(EYFP)Cos/J (ROSA26EYFP) or B6.129(Cg)-Gt(ROSA)26Sor m4(ActB–tdTomato–EGFP)Luo/J (ROSA26mTomato/mEGFP) reporter mice from the Jackson Laboratories. Ret+/EGFP mice were provided by Sanjay Jain (Washington University, St. Louis, Missouri, USA).

**Tissue preparation.** Fetal and newborn mouse kidneys were fixed in 10% (vol/vol) buffered formalin at room temperature for 24 to 48 hours. Adult mice were euthanized and transcardially perfused with PBS, followed by 10% formalin. Adult organs were further fixed for 48 hours in formalin, before processing and embedding in paraffin.

**Immunostaining.** Immunohistochemistry and immunofluorescence were performed as described previously (19, 37). The following antibodies were used: mouse anti–pan-p63 (clone 4A4, Santa Cruz; clone SFI-6, Sigma-Aldrich; and DCS, Innovative Diagnostik-Systeme), rabbit anti-ΔNp63 (26), mouse anti-CK (Sigma-Aldrich, catalog C2562, 1:200), rabbit anti-Pax2 (Invitrogen, catalog 71-6000, 1:200), chicken anti-AQP2 (Invitrogen, catalog 71-6000, 1:200), and chicken anti-Carbonic anhydrase II (CAII, green).
GFP (Abcam, catalog ab13970, 1:1000), mouse anti-E-cadherin (BD Biosciences, catalog 610181), rabbit v-H-ATPaseB1 (gift from Dennis Brown, Harvard Medical School, 1:1,000), rabbit anti-pendrin (gift from Susan Wall, Emory University, Atlanta, Georgia, USA; 1:20,000), rabbit anti-AE1 (Santa Cruz, 1:200), goat anti-AQP2 (Santa Cruz, 1:200), and goat anti-carbonic anhydrase 2 (Santa Cruz, 1:400). For immunofluorescence, image acquisition was performed using the Nikon Upright Microscope (Eclipse NI-U) and Nikon NIS-Elements Imaging Software (v4.40.00, build 1084, 64bit).

Study approval. Animal care and experiments were performed according to the guidelines of Tulane University School of Medicine for Animal Care Committee (under approved animal protocol 4341).

Author contributions
SSED and ZS conceived and designed the experiments. YL, JL, JCP, KSHS, and EG performed the experiments. SSED wrote the paper. S Signoretti and S Sinha contributed reagents and mice and revised the paper.

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