RUNNING HEAD: Tcf21 in kidney stroma cell fate

The Transcription Factor TCF21 is necessary for adoption of cell fates by Foxd1+ stromal progenitors during kidney development.

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ABSTRACT

Normal kidney development requires coordinated interactions between multiple progenitor cell lineages. The Foxd1+ stromal progenitors are critical for normal nephrogenesis and their heterogeneity is increasingly appreciated. However, the molecular mechanisms and trajectories that drive the differentiation of Foxd1+ cells toward the renal stroma, capsule, mesangial cells, renin cells, pericytes, and vascular smooth muscle cells (VSMCs) are poorly understood. Recent work has implicated Tcf21, a mesoderm-specific bHLH transcription factor critical for embryogenesis, in the development of the kidney stroma and perivascular cells. To investigate the role of Tcf21 in Foxd1+ cells, we performed single-cell RNA sequencing (scRNA-seq) on GFP+ cells from E14.5 *Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{f/f}$ kidneys (*Tcf21*-cKO) and *Foxd1*^{Cre} controls. Clustering of the entire dataset identified a large stromal population and a smaller representation of non-stromal lineages. Subclustering of stromal cells identified six populations associated with healthy kidney development: medullary/perivascular, proliferating, differentiating nephron, nephrogenic zone-associated, collecting duct-associated, and ureteric. Loss of Tcf21 resulted in a dramatic reduction in the medullary/perivascular, proliferating, nephrogenic zone-associated, and collecting duct-associated stromal subpopulations. Immunostaining confirmed that *Tcf21*-cKO has a severe constriction of the medullary and collecting duct-associated stromal space. We identified and validated a cluster unique to *Tcf21*-cKO kidneys exhibiting mosaic expression of genes from nephrogenic, proliferating, medullary, and perivascular stromal cells spanning across all pseudotime, suggesting cells halted in the midst of differentiation. These findings underscore a critical role for Tcf21 in the emergence of Foxd1+ derivatives, with loss of Tcf21 leading to a shift in stromal cell fates that results in abnormal kidney development.

NEW & NOTEWORTHY

The mechanisms responsible for the emergence of renal stromal heterogeneity has been unknown. Using scRNA-seq on Foxd1+ enriched cells from E14.5 kidneys, we identified seven molecularly distinct stromal populations and their regional association. The data suggest that the transcription factor Tcf21 regulates the adoption of fates by Foxd1+ cells that is required to form the normal milieu of stromal derivatives for the development of a kidney of normal size and function.

Keywords: Foxd1; kidney; scRNA-seq; stroma; Tcf21

INTRODUCTION

Work that has spanned over seven decades increased our understanding of molecular mechanisms in kidney development and has been informing new regenerative based solutions for drug screening, disease modelling, and renal replacement [1,2]. The developing mammalian kidney is composed of four progenitor lineages: nephron (Six2+ cells), ureteric (Hoxb7+ cells), stromal/interstitial (Foxd1+ cells), and endothelial (c-kit+ cells) [3,4]. During kidney development, these progenitor populations signal to each other to ensure the ongoing expansion of the kidney to an organ of normal size and function [5,6]. The forkhead box D1 (Foxd1+) progenitors give rise to a diverse array of cell types including most of the renal stroma, vascular smooth muscle cells (VSMCs), pericytes, mesangial cells, renin-, and erythropoietin-producing cells [7,8]. Conventionally, renal stroma is divided into three anatomical regions: cortical, medullary, and papillary [9]. Recent research,

however, has identified twelve distinct anatomical positions within the stroma in mice, each characterized by unique cell types, highlighting its heterogeneity [10]. Foxd1+ cells play critical roles in branching morphogenesis, nephron development, and vascular patterning [11], possibly by promoting maturation of neighboring epithelial and endothelial cells [12-15]. Despite these insights, understanding the molecular mechanisms through which the stroma influence non-stromal cells and guide the differentiation of Foxd1+ cells into distinct stromal populations is essential for normal kidney development, yet remains incomplete.

TCF21 is a mesoderm-specific transcription factor of the bHLH family that forms homo/heterodimers upon binding to its target DNA sequence. Originally named Pod1 [16], epicardin [17], or capsulin [18,19], Tcf21 is expressed in the developing urogenital, cardiovascular, respiratory, and gastrointestinal systems during embryonic development. Tcf21 has been recently implicated as a causal gene for coronary artery disease in humans [20,21]. Within the kidney, Tcf21 has been implicated in the formation of the renal stroma and perivascular cells, influencing the structural integrity and function of the organ [22]. To investigate the role of Tcf21 on the development of kidney stroma, we performed scRNA-seq on normal and Tcf21-deficient Foxd1+ kidney cells from E14.5 mouse embryos, an early stage of nephrogenesis.

MATERIALS AND METHODS

Mouse strains and embryo staging

All mouse experiments were approved by the Animal Care Committee at the Center for Comparative Medicine of Northwestern University (Evanston, IL) and were performed in accordance with institutional guidelines and the NIH Guide for the Care and Use of Laboratory[6] Animals. For gestational age, noon of the day on which the mating plug was observed was designated E0.5. Sex was determined before analysis and samples were pooled accordingly. The following mice were used in the studies described: *Tcf21*$^{flox/flox}$ was created as previously described [23], *Foxd1-eGFPCre* (JAX Stock #012463), Rosa26Tomato (JAX Stock #007909). Genotyping was performed with Quick-Load Taq 2X Master Mix (M0271L, BioLabs) on 2% agarose gel for the PCR bands.

Histology and immunohistochemistry

Mouse embryo dissections were performed at the indicated time-points. Samples were fixed with 10% Neutral buffered formalin or 4% paraformaldehyde overnight, embedded in paraffin, and sectioned into 5µm by the Mouse Histology and Phenotyping Laboratory of Northwestern University. Sections were deparaffinized in xylene and ethanol and boiled in citrate buffer for antigen retrieval. Sections were then treated with 1% donkey serum with 0.3% TritonX100 in PBS for 30 minutes at room temperature before incubating with primary antibodies at 4°C overnight. Sections were washed in PBS and incubated with anti-GFP antibody (A11122, Life Tchnologies, 1:1000), anti-EMCN antibody (ab106100, Abcam, 1:250), and anti-ACTA2 antibody (A2547, Sigma, 1:400). Alexa Fluor 488 and 647 fluorescence-conjugated secondary antibodies were incubated for 90 minutes at room temperature.

Preparation of single cells from E14.5 mouse embryos

E14.5 *Foxd1*^{Cre}*;Rosa26*^{mTmG}*;Tcf21*$^{f/f}$ and *Foxd1*^{Cre}*;Rosa26*^{mTmG}*;Tcf21*$^{+/+}$ mouse embryos were excised and kidneys were dissected and washed in cold PBS without calcium or magnesium. We collected cells pooled from n=4 control males, n=2 control females, n=1 *Tcf21*-cKO male, n=3 *Tcf21*-cKO females. Cell dissociation was performed as previously described [24]. Briefly, the kidneys were minced and digested with freshly prepared mix of Collagenase type 2 (Worthington, LS004174, 2.5mg/ml), DNase I (Worthington, LS002058, 125u/ml), and Licheniformis Protease 7.5mg/ml by incubating for 40 min at 6 deg Celsius with manual dissociation via pipetting every 5 minutes. Digestion was inactivated using stop solution of 20% FBS in PBS (Ca-, Mg-). After pelleting the cells at 1250 rpm for 7 min, cells were resuspended in 1 ml of DMEM (Dulbecco's Modified Eagle Medium, Gibco, Netherlands) + FBS 2% and passed through a 40uM" strainer (Scienceware Flowmi, H13680-0040) into DNA LoBind tubes (Eppendorf, 022431005)). Cells were resuspended in 500 µl DMEM + FBS 2% and filtered two more times through to a 5 ml polypropylene round bottom tube (Falcon™ 352063). Cells were sorted at the Flow Cytometry Cell Sorting core at Northwestern University on a BD FACSAria SORP system and the BD FACSymphony S6 SORP system. GFP positive cells were collected and resuspended in PBS with 1%BSA (Bovine Serum Albumin) for immediate use. Samples of shared sex and genotyping were pooled leading to 4 samples.

Initial Processing of Single-cell RNA-seq

Single cells were processed using the 10x Genomics Chromium 3' Kit version 3 and sequenced on an Illumina NovaSeq with a target of ~400,000,000 reads per sample. Raw sequencing data was demultiplexed and processed using Cell Ranger v6.1.0 and a custom reference combining the mm10 transcriptome with the eGFP and tdTomato RNA sequences. Filtered count metrics from each sample were loaded as a Seurat object (v4.0.2). Genes were retained in the analysis only if expressed in at least three cells. Cells exhibiting fewer than 2000 or more than 8000 gene features were filtered out of the subsequent

analysis. Moreover, cells with mitochondrial content exceeding 15% or with hemoglobin gene (Hba and Hbb) expression above 1%, were also discarded. Following this, Scrublet (v0.2.2) was used to identify cells likely to be doublets, applying 25 principal components for each sample. Across all samples, an expected doublet rate threshold of 7.6% (based on loading of approximately 16,000 cells) was set to ensure thorough and consistent doublet identification leading to 2701 cells marked for removal. After filtering, all samples were merged into a single Seurat object. Estimated cells numbers and initial quality control (QC) statistics are given in the table below:

Sample	Pre-QC Cell #	Median Genes	Median UMIs	% Reads in Cell	Post-QC Cell #
Control Female	11,049	3,570	12,569	95.5	9526
Control Male	11,995	3,946	15,391	94.7	9679
Mutant Female	7,867	4,468	18,576	93.2	5830
Mutant Male	9,543	4,387	19,294	94.0	7426
Total					**32,461**

Cell Type Annotation

SCTransform was applied on 3000 variable genes for data normalization and scaling, with UMI count and percent mitochondria set as variables to regress. Principal component analysis (runPCA) was performed across all samples and the first 25 PCs were selected for Uniform Manifold Approximation and Projection (UMAP). Clustering (FindClusters) was also run with 25 PCs and a resolution parameter of 0.25. To identify *de novo* markers for the resulting 13 clusters, FindMarkers was run using a two-sided Wilcoxon rank-sum test (min. pct = 0.25) with adjusted p-value calculated using Benjamini-Hochberg method. Individual clusters were grouped together based on annotated cell type for further analysis.

Stromal Subclustering

To identify subpopulations within stromal clusters, we selected all cells from the clusters annotated as stroma (n = 22,355). Cells exhibiting more than 10% mitochondrial content were excluded to eliminate stressed or dying cells that could skew the analysis.

Sample	All Stromal Cells	Median Genes	Median UMIs	Filtered Stromal Cells
Control Female	8912	3577	12558	8897
Control Male	6724	3889	14559	6689
Mutant Female	4939	4668	19993	4934
Mutant Male	1780	4046	14959	1777
Total	**22,355**	**3922**	**14,644**	**22,297**

The remaining 22,297 cells were renormalized using scTransform with 3000 variable genes and regressing on the difference between S and G2M score (CellCycleScoring) as recommended by Seurat to limit the effect of cell cycle on clustering while maintaining differentiation-associated trends. UMAP and clustering (FindClusters) were performed with the first 15 principal components and resolution at 0.20. *De* novo markers across stromal clusters were identified as described above for cell types. Transcriptional signatures of stromal compartments were calculated using AddModuleScore on curated markers based on publicly available sources [10,25], Allen Developing Mouse Brain Atlas (https://developingmouse.brain-map.org/), GUDMAP [26,27], and our previous studies [22] (**Suppl Fig. S3**). To compare our annotations to previously published clusters (Combes et al), we calculated the fraction of the top 20 published markers that overlapped our list of *de novo* markers by cluster.

GenePaint

The spatial expression of representative key genes in control E14.5 mouse kidney was examined using *GenePaint* gene expression atlas (https://gp3.mpg.de/).

Comparison of Stromal Cell in *Tcf21*-cKO and Control

Before comparing stromal progenitors across genotype, each sample was subsampled to 1777 stromal cells to match the lowest sample and ensure even numbers across sexes. The scProportionTest algorithm (https://github.com/rpolicastro/scProportionTest) was used to calculate p-values for differences in composition between *Tcf21*-cKO and control conditions using a permutation test. Confidence intervals were provided through bootstrapping. Differential expression analysis between conditions was performed using FindMarkers on all stromal cells in *Tcf21*-cKO vs. control (min.pct = 0.25). Genes with log fold change ≥ 0.25 and with p-adjusted value less than ≤ 0.05 were defined as significantly increased or decreased in expression. Enriched GO terms for genes that exhibited significantly increased or decreased expression in *Tcf21*-cKO were determined using the R package clusterProfiler (https://github.com/YuLab-SMU/clusterProfiler) and compared all 28,891 genes expressed in the dataset as background.

Trajectory analysis

To construct a pseudotime trajectory representing healthy differentiation, cells from control samples (n = 15,579, excluding 7 outlier cells from the ECM-expressing cluster) were isolated from the stromal population. This subset of cells was normalized using scTransform with regression by cell cycle score difference as above (S-G2M). Then, RunPCA and RunUMAP (dim=15) was performed. The two-dimensional representations was converted to be compatible with Monocle v3 [28,29]. Clustering was performed in Monocle using the cluster_cells function with default parameters. To build the underlying trajectory, learn_graph was run with parameters close_loop=FALSE and ncenter=310 (chosen based on calculating 2% of the number of cells). The starting node was chosen to reflect the Nephogenic zone stroma using order_cells(). The pseudotime for each cell was calculated by picking up the one open circle (set as pseudotime =0) in the nephrogenic zone cluster. The function graph_test() was used to identify co-regulated genes across pseudotime with the autocorrelation approach ("principal_graph" parameter). Significant genes were defined by q value < 0.05. Genes were grouped into trajectory-based modules using find_gene_modules() with default parameters testing a range of resolution values from 10^{-6} to 10^{-1} and the aggregate_gene_expression was used to calculate module expression. The pseudotime calculated in the control samples was binned into discrete intervals (bins) of 3 units each, ranging from 0 to 30. To transfer the pseudobins from control cell population, the *Tcf21*-cKO samples were first isolated from the stromal population and than processed using the same method done for control samples. To transfer the pseudobins from the control sample to *Tcf21*-cKO samples, FindTransferAnchors() function was used to find the anchors between control and the *Tcf21*-cKO using the PCA as a dimenionality reduction technique. Later these anchors were transferred onto the *Tcf21*-cKO samples using TransferData() function. further we used MapQuery() function to project the *Tcf21*-cKO cells onto the PCA and UMAP space of the control samples ensuring the Transfer of pseudobins alongside with it by providing a pseudobin as a reference data. To calculate enrichment of each trajectory-based module in the Emcn-expressing cluster in *Tcf21*-cKO samples, the percent of cells with score greater than 0 in this cluster compared with the rest of the sample was calculated. These numbers were used in a hypergeometric test to determine the p-value for enrichment.

RESULTS

Single-cell profiling of E14.5 *Foxd1*GFP+ cells identifies diverse cell populations within the developing kidney

To investigate the role of Tcf21 in the Foxd1+ stromal progenitors and their derivative cells at a single-cell level, we purified GFP+ cells from male and female *Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{f/f}$ (*Tcf21*-cKO) and littermate controls *Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{+/+}$ via flow cytometry. In this model, *Tcf21* gene is permanently deleted, tdTomato expression is terminated, and GFP expression is maintained in all the progeny of Foxd1-expressing cells. We obtained 13,256 cKO and 19,205 control cells after QC (**Suppl Fig. S1a-b**). We defined 13 clusters using an unsupervised approach (see Methods) and assigned cell lineage annotations based on *de novo* markers and canonical gene expression (**Suppl Fig. S1c-d**). This analysis identified a large population of stromal cells (Foxd1+ Meis1+ Pdgfra+) as well as representation of the nephron progenitor cell (NPC) lineage (Six2, Cited1, Crym), and significantly smaller populations of ureteric epithelium (Ret, Wnt 11, Gata3), immune cells (Fcer1g, Lyz2), and neurons cells (Tubb3, Map2) (**Fig.1a-b, Suppl Fig. S1e**). Cycling genes were expressed across cell lineages (**Suppl Fig. S1f**). We confirmed that GFP expression was robust in both stromal and non-stromal cells, though Foxd1 expression was largely limited to the stromal lineage (**Fig. 1c**). Low expression of tdTomato and depleted expression of Tcf21 in *Tcf21*-cKO cells validate the activity of cre recombinase in these cells (**Fig. 1c, Suppl Fig. S1g**). Non-stromal GFP+ cells likely represent early transient expression of Foxd1 before the onset of lineage boundaries. All cell types were represented in both males and females of *Tcf21*-cKO and control samples, however the number and proportion of stromal cells was lower in sex-matched mutant samples (**Fig. 1d**, **Suppl Fig. S1h**).

E14.5 stromal progenitors express genes associated with different cell fates

Next, we sought to explore the heterogeneity within the developing stromal cell populations by sub-clustering cells annotated as stroma. We defined seven stromal subpopulations (numbered 0-6) in both *Tcf21*-cKO and controls with distinct gene expression profiles based on *de novo* marker expression (**Fig. 2a-b, Suppl Fig. S2a-b**). All clusters expressed similar levels of GFP and low level expression of tdTomato although expression of *Foxd1* and *Tcf21* was variable (**Suppl Fig. S2c**). To annotate these clusters, we created modules associated with different stromal subpopulations based on canonical markers from the literature (**Suppl Fig. S3**) and visualized their expression along with canonical cell cycles genes (**Fig. 2c**). Based on module expression and *de novo* genes markers, we annotated Nephrogenic zone associated stroma; Proliferating stromal cells; Medullary/Perivascular associated stroma; Collecting duct associated stroma; stroma of the Differentiating nephron; and Ureteric stroma. To confirm our annotations, we compared expression of key genes in our single-cell data vs. spatial expression from the *GenePaint* gene expression atlas (**Fig. 3**). Our annotations largely agreed with previously published stromal clusters during kidney development (Combes et al [25]), with subtle differences (**Suppl Fig. S2d-e**). Our annotated Differentiating nephron cluster (cluster 3) more closely resembles the published Ureteric cluster. However, the lower levels of *Tbx18, a gene critical to* early separation of the ureteric mesenchymal lineage from other mesenchymal lineages [11 30], in these cells compared with those in cluster 6 did not support its classification as Ureteric. Moreover, *de novo* markers with unique expression in cluster 6 demonstrated high spatial specificity to the ureteric zone (**Fig. 3).** Instead, we chose the annotation of Differentiating nephron for cluster 3 due to more diffuse expression of associated markers (**Fig 3**). Additionally, we annotated cluster 2 as Emcn-expressing based on unique expression of the gene Endomucin (*Emcn*), further discussed in the following section (**Fig. 2a-b, Suppl Fig. S2f**).

<u>Tcf21 is required for emergence of stromal subpopulations during kidney development</u>

Next, we sought to determine how stromal progenitor cells in *Tcf21*-cKO have deviated from controls using a downsampled dataset to control for sex and cell number (see Methods). Strikingly, Nephrogenic zone, Proliferating, Medullary/Perivascular, and Collecting duct associated stroma clusters were dramatically reduced in *Tcf21*-cKO compared to control (**Fig. 4a-c**). Conversely, Differentiating nephron-associated and ureteric stroma clusters showed moderate increases in *Tcf21*-cKO, although this may be due to reduced total stromal cell numbers. Notably, the most profound change was that the Emcn-expressing cluster was virtually exclusive to *Tcf21*-cKO kidneys, with only a few cells (7 cells) of shared transcriptional profile identified in stromal cells of controls (**Fig. 4a-c**).

In parallel, we performed differential gene expression analysis of the whole stromal compartment between *Tcf21*-cKOs and controls (**Suppl Table S3b**). We identified 214 genes up-regulated and 64 genes down-regulated in *Tcf21*-cKO compared with control cells (**Fig. 4d**). Tcf21 is among the most down-regulated genes, as expected, while genes associated with connective tissue development, extracellular matrix organization, epithelial cell proliferation, and WNT pathway, such as Tgfb2, Sparcl1, Postn, and Rgs5, were upregulated (**Fig. 4e, Suppl Fig. S4a**). In order to determine how the Emcn-expressing cells fit into healthy stromal cell differentiation, we ran Monocle on control stromal cells to estimate the underlying pseudotime trajectory assuming Nephrogenic zone associated cells represented the most progenitor-like subpopulation. By transferring control-based pseudotime to *Tcf21*-cKO cells, we found that the Emcn-expressing cluster comprised cells spanning across all pseudotime (**Fig. 4f-g**). Moreover, when we defined modules of co-regulated genes along the control trajectory, we found that specific cell subsets within the Emcn-expressing cluster expressed genes associated with a variety of other subpopulations including nephrogenic (module 18), proliferating (module 20), and medullary/perivascular (module 14) (**Suppl Fig. 4c-d**). Taken together, these results suggest that the *Tcf21*-cKO is associated with the emergence of cells that are halted in the midst of differentiation.

To spatially define the defect in the stromal lineage of the *Tcf21*-cKO, we performed lineage-tracing of Foxd1 (GFP+) in *Foxd1*^{Cre}*;Rosa26*^{mTmG}*;Tcf21*$^{f/f}$ and control mice from E14.5 to E18.5. *Tcf21*-cKO demonstrated a marked constriction of the medullary stroma and the stromal space adjacent to the collecting ducts compared with controls (**Fig. 5a**). Additionally, immunostaining for endomucin, a marker of the stromal population exclusively expressed in *Tcf21*-cKO, confirmed robust expression solely in the stromal lineage of *Tcf21*-cKO kidneys at E14.5 and E16.5 (**Fig. 5b**). Furthermore, staining with alpha smooth muscle actin (Acta2), which marks medullary stromal cells ([10] and [22]), demonstrated a loss of Acta2-expressing stroma and the emergence of Emcn-positive stromal cells in *Tcf21*-cKO kidneys, supporting a population shift (**Fig 5c**). Taken together, these data support the scRNA-seq analysis results above and indicate a requirement for Tcf21 in the emergence of specific derivatives of the stromal lineage during kidney development (**Fig. 5d**).

DISCUSSION

Our findings underscore the critical role of Tcf21 in the differentiation and fate determination of Foxd1+ stromal progenitors during kidney development. Using scRNA-seq and detailed sub-clustering analysis, we identified six distinct stromal populations within the healthy developing kidney. The deletion of Tcf21 led to a significant reduction in specific stromal populations, including medullary/perivascular stroma, proliferating stroma, nephrogenic zone-associated stroma, and collecting duct-associated stroma. Concurrently, a novel stromal population emerged in Tcf21-deficient kidneys,

characterized by high expression of Endomucin, a gene from the sialomucin family typically expressed in endothelial cells of the vasculature. This new population was annotated as Endomucin-expressing stroma. Our trajectory analysis suggested that this novel stromal population represents cells arrested in their differentiation process.

The kidney stromal compartment is known for its high heterogeneity, with Foxd1+ progenitors giving rise to diverse cell types such as fibroblasts, VSMCs, pericytes, renin cells, and mesangial cells. Previous studies have highlighted the pivotal roles of Foxd1+ cells in branching morphogenesis, nephron development, and vascular patterning. However, until now, the molecular mechanisms guiding the differentiation of these cells into distinct stromal lineages have been poorly understood. The medullary and collecting duct stromal compartments are particularly crucial for the kidney's structural integrity and function. The medullary stroma/interstitium is essential for maintaining the osmotic gradient required for urine concentration, while the collecting duct-associated stroma plays a vital role in the development and function of the collecting duct. The loss of these stromal populations in *Tcf21*-cKO was associated with disrupted collecting duct organization and a urine concentrated defect after birth [22]. Mechanistically, Tcf21 may facilitate Foxd1+ cell proliferation at E14.5, which aligns with our observations at E16.5 and E18.5, as well as with reports of Tcf21's inhibition of cell cycle arrest-promoting factors [31].

The identification of a distinct stromal population exclusively present in Tcf21-depleted Foxd1+ cells, characterized by unique expression of endomucin, and by upregulation of genes marking vascular smooth muscle cell fate (*Rgs5*) and extracellular matrix components (*Sparcl1*), suggests that Tcf21 may play a critical role in VSMC formation during kidney development. Our trajectory analysis indicates that this novel stromal population represents cells arrested in their differentiation process, drawing parallels to Tcf21's known role in VSMC phenotypic modulation in human coronary artery disease [32]. Histological analysis identified the novel Endomucin-expressing stroma in *Tcf21-cKO*, which was associated with a concurrent decrease in medullary Acta2-expressing stromal cells, supporting a population shift. This aspect of Tcf21 function may shed light on how Tcf21 influences VSMC differentiation and vascular integrity, consistent with our previous finding of disrupted arterial tree in *Foxd1Cre;Tcf21-cKO* [22].

Interestingly, we observed an increase in ureteric-associated stroma and differentiating nephron-associated stroma in *Tcf21-cKO* kidneys, suggesting potential compensatory mechanisms or shifts in cell fate in response to Tcf21 loss. Lineage tracing for Foxd1 revealed a global reduction in the stromal space at E14.5, while the medullary stroma was predominantly affected at E16.5 and E18.5, consistent with compensation. High Tcf21 expression in proliferating, medullary/perivascular, and collecting duct-associated stroma, compared to nephrogenic zone cells, suggests that Tcf21 action occurs upon Foxd1+ cell priming. Conversely, low Tcf21 expression in ureteric stromal clusters and the independence of these populations from Tcf21 indicate that the emergence of stromal cells surrounding the ureter is not dependent on Tcf21 expression in Foxd1+ cells.

Gene Ontology enrichment analysis between *Tcf21-cKO* and control stromal cells revealed increased expression in genes involved in connective tissue development, epithelial cell proliferation, and WNT signaling pathways. We propose a model in which Tcf21 plays a critical role in the differentiation of Foxd1+ stromal progenitors during kidney development by regulating the emergence and composition of distinct stromal populations, thereby ensuring proper kidney development and function (**Fig. 5d**).

The use of single-cell RNA-seq to understand the role of Tcf21 in kidney development has some limitations. First, although Foxd1 is associated with stromal progenitors, our samples included GFP+ non-stromal cells. This may be due to leakiness in the cre-flox system or, more likely, transient expression of Foxd1. Expression of Foxd1 in NPC has been reported by others [8,33-36] and is thought to stem from a shared signature during early nephrogenesis [25]. Additionally, autofluorescence generated by non-stromal cell types may have overlapped with the GFP channel. Tcf21 expression was detected in the stromal and NPC lineages, consistent with previous reports [37]. Moreover, the cre model may disrupt normal expression of Foxd1, although the pooling of mice and Foxd1-cre controls alleviate this concern. Finally, this study is limited by a snapshot of a single time point of gene expression, which restricts cell annotation and interpretation of definitive cell fates.

Beyond kidney development, our findings suggest broader implications for Tcf21 in mesodermal development, including the heart and respiratory systems, expanding our understanding of Tcf21's role in these tissues and its potential impact on fibrotic diseases characterized by excessive ECM deposition. Further studies are required to identify transcriptional networks, direct targets, and signaling pathways in Tcf21 regulatory networks in the regulatory of Tcf21 in Foxd1+ cells.

DATA AVAILABILITY

Data will be uploaded to the Gene Expression Omnibus upon acceptance of the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S4:

Suppl Fig. S1: Single-cell RNA-seq analysis of Foxd1GFP+ sorted cells from control and *Tcf21*-cKO kidneys.

Suppl Fig. S2: Single-cell RNA-seq analysis of stromal cells

Suppl Fig. S3: Stromal subpopulation markers for module scores
Suppl Fig. S4: Comparison of stromal cells between *Tcf21*-cKO and controls

Supplemental Tables S1–S4:
Suppl Table S1: a. All cluster markers. b. Cell type markers
Suppl Table S2: a. Stromal subpopulation markers. b. Marker overlap with Combes et al
Suppl Table S3: a. Downsampled cell numbers by stromal cluster by sample b. Differential gene expression output c.Go term output
Supp Table S4. a. Monocle module genes. b. Number of cells expressing modules

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DISCLOSURES
Susan E. Quaggin holds patents related to therapeutic targeting of the ANGPT-TEK pathway in ocular hypertension and glaucoma and owns stock in and is a director of Mannin Research. S. Quaggin also receives consulting fees from AstraZeneca, Janssen, the Lowy Medical Research Foundation, Roche/Genentech, Novartis, and Pfizer and is a scientific advisor or member of AstraZeneca, Genentech/Roche, JCI, the Karolinska CVRM Institute, the Lowy Medical Research Institute, Mannin, Novartis and Goldilocks. DRW received consulting fees from Pfizer during the duration of this project. All other authors have nothing to disclose.

FIGURE LEGENDS
Figure 1: scRNA-seq analysis of the Foxd1-GFP+ sorted cells from E14.5 embryonic mouse kidneys of Tcf21-conditional knockout (*Tcf21*-cKO) and controls. (a) UMAP plot of 32,461 individual cells combined from male and female *Tcf21*-cKO and control kidneys coloured by cell type annotation (b) DotPlot illustrating expression of canonical genes across cell types. Size of dot illustrates percent of cells expressing the gene and colour scale indicates relative expression across cell types. (c) Feature plots of EGFP, Foxd1, tdTomato, and Tcf21 gene expression ($\log_2$ transformed). (d) UMAP plot of samples by experimental group and sex.. See **Suppl Fig. S1** for number of cells per experimental group.

Figure 2: Stromal lineage sub-clustering identifies populations of distinct cell fate within the developing mouse kidney. (a) Sub-clustering of stromal cells (n=22,297) from E14.5 Foxd1GFP+ kidney cells (stromal cluster in **Fig. 1a**) identifies seven transcriptionally distinct populations and their suggested annotation. (b) Heatmap depicting the relative expression of 5 of the top de novo marker genes for each cluster across individual cells. Column color coding is consistent with **Fig. 2a.** (c) Feature plot of the module score distribution in UMAP space based on canonical gene signatures (listed in **Suppl Fig. 3**).

Figure 3: Spatial annotation of stromal clusters of control and *Tcf21* cKO. (a) Featureplot showing the $\log_2$-transformed gene expression of markers for Nephrogenic zone stroma (*Foxd1, Crabp2, Gdnf, Aldh1a2*), Medullary and Perivascular stroma (*Pdgfrb, Ace2, Rgs5, Heyl*), Collecting duct stroma (*Penk, Pfn2, Tcf21, Thy1)*, stroma of the Differentiating nephron (*Smoc2, Igf1, Dcn, Fbln5*), and Ureteric stroma (*Tbx18, Syt1, Npy1r)*. (b) Expression of the same marker genes by *in situ* hybridization in control E14 mouse kidneys as per *GenePaint* gene expression atlas (https://gp3.mpg.de/, cropped images

from whole mount). Includes schematic illustration of the spatial location of each stromal population in the developing kidney. Cluster 0 (Medullary and Perivascular stroma), cluster 4 (Nephrogenic zone stroma), cluster 5 (Collecting duct stroma), cluster 3 (stroma of the Differentiating nephron), and cluster 6 (Ureteric stroma).

Figure 4: Tcf21 is critical for the development of specific stromal cell fates. (a) UMAP of Foxd1GFP+ 7108 stromal cells (downsampled to equally reflect 4 samples) from male and female E14.5 control and *Tcf21*-cKO mouse kidneys (*Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{+/+}$ and *Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{f/f}$, respectively). (b). Stacked bar chart indicating subpopulation composition by sample. Percent of each subpopulation in a sample are given. (c) Relative differences in cell proportions for each cluster between the *Tcf21*-cKOs versus controls. Dashes vertical lines mark absolute Log_2 fold change (FC) > 1 compared with the control. All changes in proportion were significant after FDR was applied (scproportion permutation test; n = 3554/group; Supp Table 4A). (d) Volcano plot of differentially expressed genes (DEGs) between all stromal cells in *Tcf21*-cKOs versus controls defined by log_2(fold change) >0.25 and adjusted p-value <0.05. Red and blue dots denote significantly up- and down-regulated differential genes, respectively. (e) DotPlot of GO Terms significantly enriched among up-regulated (top panel) and down-regulated (bottom panel) DEGs. X axis denotes the overlap ratio (# genes in overlap/# genes in GO term, size of dot denotes the #genes in overlap, and color scales deotes the -log_{10} adjusted p-value (p-adj). (f) Feature plot of stromal cells with color-coding based on pseudotime bins. g) Box and whiskers plot depicting the range of pseudotime by bin in each stromal cluster.

Figure 5: Lineage tracing and population shift in Foxd1+ stromal cells of *Tcf21*-cKO kidneys. (a) Immunofluorescence (IF) staining of *Foxd1*^{Cre};*Rosa26*^{mTmG};*Tcf21*$^{f/f}$ and control kidneys using an anti-GFP antibody shows a significant reduction in the stromal space in *Tcf21*-cKO at E14.5, consistent with scRNA-seq findings. Lineage tracing at E16.5 and E18.5 indicates that the loss of stromal space persists in medullary stroma and stroma adjacent to the collecting ducts in *Tcf21*-cKO kidneys (dotted line). Scale bar 500 μm. (b) IF staining of EMCN reveals the presence of the novel Endomucin-positive stromal cells (arrowheads) in *Tcf21*-cKO at E14.5 and E16.5. In contrast, Endomucin expression is restricted to the endothelium and absent in stromal cells in control kidneys at E16.5 (arrow). (c) Alpha smooth muscle beta-actin (Acta2)-positive stromal cells in controls (arrowheads top) are lost in *Tcf21*-cKO at E16.5, and E18.5. This loss coincides with the appearance of Endomucin-positive stromal cells (arrowheads bottom), supporting the hypothesis of a populations shift. (d) Anatomical mapping of main kidney progenitor lineages, including stroma, endothelial, ureteric bud, and nephron progenitors, identified in initial scRNA-seq clustering (left). Anatomical mapping of stromal (Foxd1+) cell subpopulations identified in scRNA-seq stromal subclustering (right). Control samples showed six stromal populations (nephrogenic zone-associated, proliferating, medullary/perivascular, collecting duct-associated, differentiating nephron-associated, and ureteric stroma). In contrast, *Tcf21*-cKO samples exhibited a loss of nephrogenic zone-associated, proliferating, medullary/perivascular, collecting duct-associated stroma, along with the emergence of a novel Endomucin-expressing stromal cell population.

Supplemental Figure S1: Single-cell RNA-seq analysis of Foxd1GFP+ sorted cells from control and *Tcf21*-cKO kidneys. (a) Violin plots showing number of unique molecular identifiers (UMIs), number of genes, and percentage of mitochondrial genes from control and *Tcf21*-cKO E14.5 kidney samples. (b) UMAP plot showing the singlets and doublets assignment in control and *Tcf21*-cKO mice. Doublets were removed from further analysis (c) Heatmap depicting the relative expression of the top 5 *de novo* marker genes per cluster. Column color coding is consistent with **Suppl Fig. 1d.** (d) UMAP plot showing the unbiased clustering of cells from control and *Tcf21*-cKO mice. Thirteen clusters were defined and annotated as stromal (clusters 0,1,2,5,6,7), nephron progenitor cells (NPC) (clusters 3,4,8), epithelial (cluster 9), endothelial (cluster 11), immune (cluster 10), or neuron (cluster 12). (e) Violin plots showing log_2-transformed gene expression of the pan-stromal markers *Pdgfra* and *Meis1* across clusters. (f) UMAP plot of control and *Tcf21*-cKO cells colored by cell-cycle stage estimated using CellCycleScoring. (g) Violin plot showing log_2-transformed Tcf21 gene expression across clusters for control (blue) and *Tcf21*-cKO (red) cells. (h) Stacked bar plot displaying number of cells for main cell types separated by experimental group and sex.

Supplemental Figure S2: Single-cell RNA-seq analysis of stromal cells. (a) Violin plots showing number of unique molecular identifiers (UMIs), number of genes, and percentage of mitochondrial genes from control and *Tcf21*-cKO stromal subpopulations. (b) UMAP plot showing the stromal subpopulation annotations on the UMAP from Figure1 (c) Violin plot showing log_2-transformed gene expression of EGFP, Foxd1, tdTomato, and Tcf21 across stromal subpopulations for control (blue) and *Tcf21*-cKO (red) samples. (d) Heatmap depicting the expression of stromal gene markers from Combes et al. [25] across our stromal subpopulations. Color scale represents log_2 fold change in in expression in the given subpopulation compared with all others. (e) Heatmap depicting the proportion of Combes et al [25] top 20 stromal gene markers that overlap *de novo* markers for each of our subpopulations. (f) Featurel plot of log_2-transformed gene expression of Emcn across

stromal cells. (g) Violin plot showing log_2-transformed Emcn gene expression across stromal subpopulations for control (blue) and *Tcf21*-cKO (red) cells.

Supplemental Figure S3: Stromal subpopulation markers for module scores. List of markers for pan-stroma and six distinct populations based on the literature references. These were used to calculate module scores for **Fig. 2c**.

Supplemental Figure S4: (a) Violin plots showing log_2-transformed gene expression of up-regulated genes (Tgfb2, Sparcl1, Postn, Rgs5) across stromal subpopulations for control (blue) and *Tcf21*-cKO (red) cells. (b) Violin plots showing log_2-transformed gene expression of down-regulated genes (Snai2, Ifitm1, Dkk1, Acta2) across stromal subpopulations, for control (blue) and *Tcf21*-cKO (red) samples. (c) Heatmap depicting the relative expression of 23 trajectory-based modules of co-regulated genes across control cells in each stromal subpopulation. On the right is shown the log_2 enrichment of cells expressing these modules in the Emcn-expressing cluster compared with the rest of Tcf21-cKO stromal cells. * indicates p-value<0.05 by hypergeometic test. (d) Feature plot showing module scores across stromal cells split by control (left) and *Tcf21*-cKO (right) for module18 (nephrogenic zone), module20 (proliferating), and module14 (medullary/perivascular).

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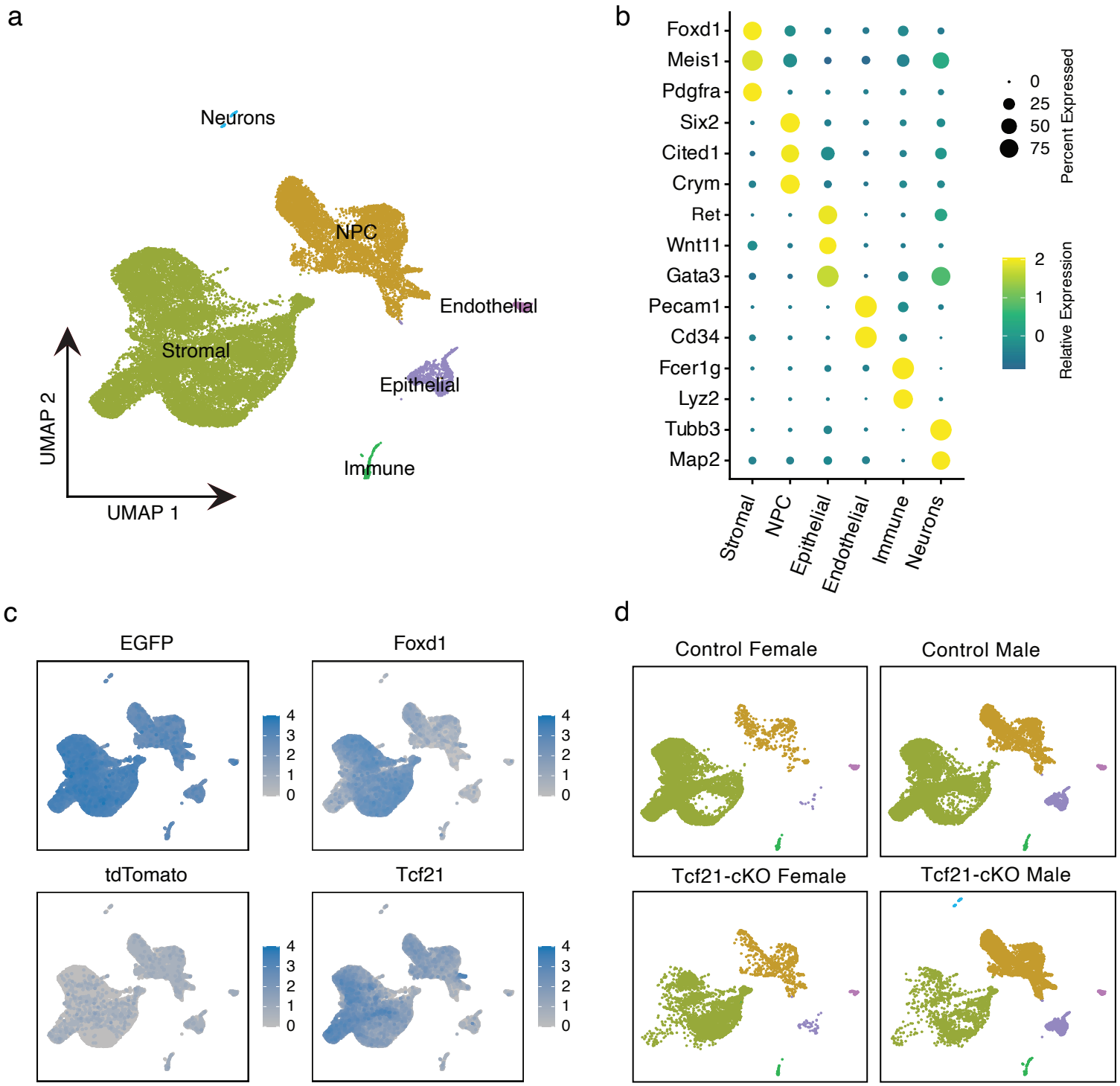
Fig 1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Percent Expressed
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Foxd1
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Fig 2

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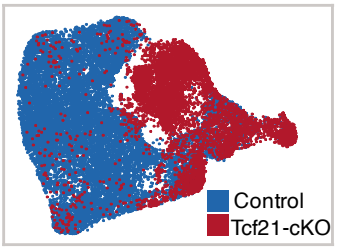
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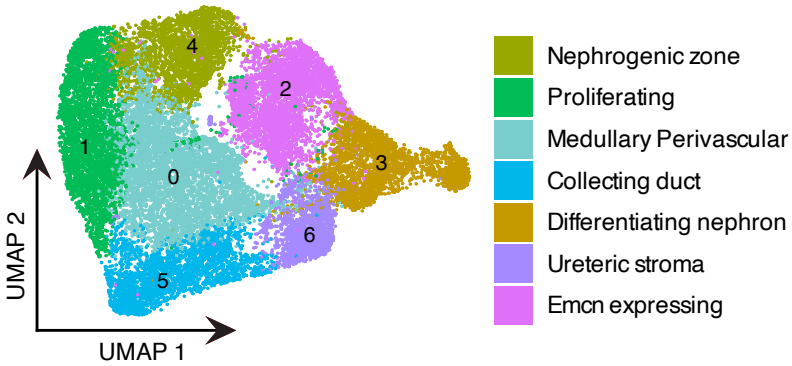
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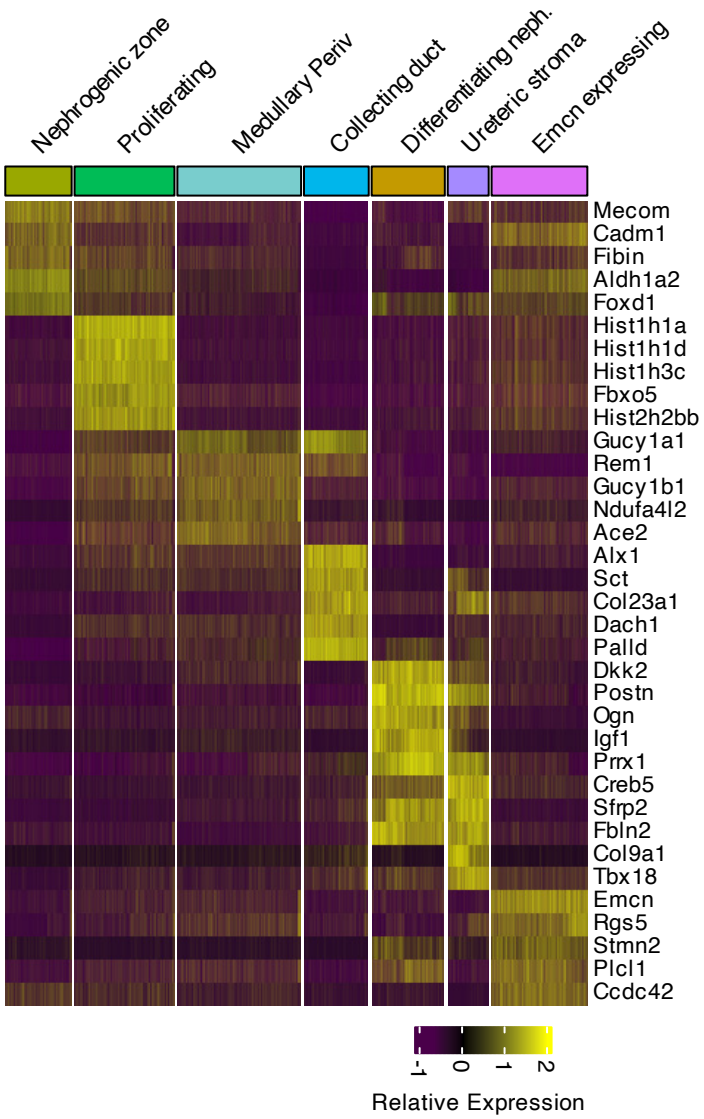
Medullary Perivascular
Collecting duct
Differentiating nephron
Ureteric stroma
Emcn expressing

b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Foxd1
Hist1h1a
Hist1h1d
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Rem1
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Alx1
Sct
Col23a1
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Sfrp2
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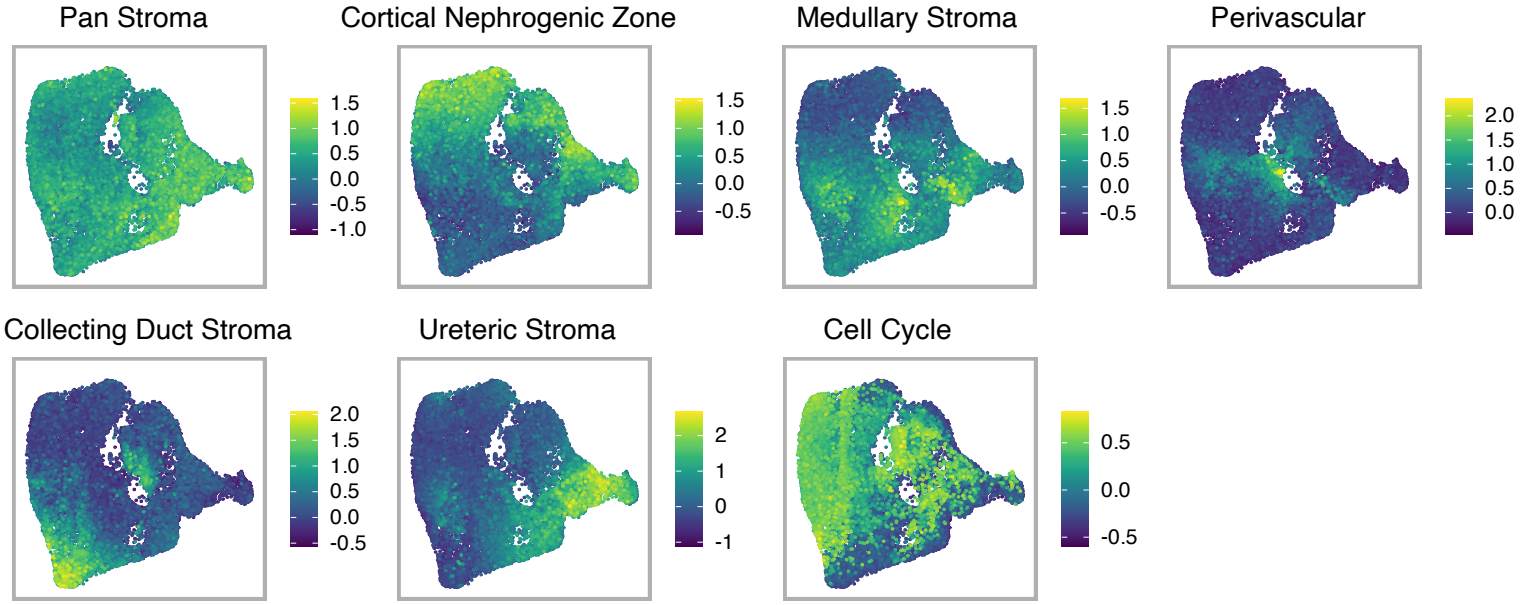
Relative Expression

c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Collecting Duct Stroma
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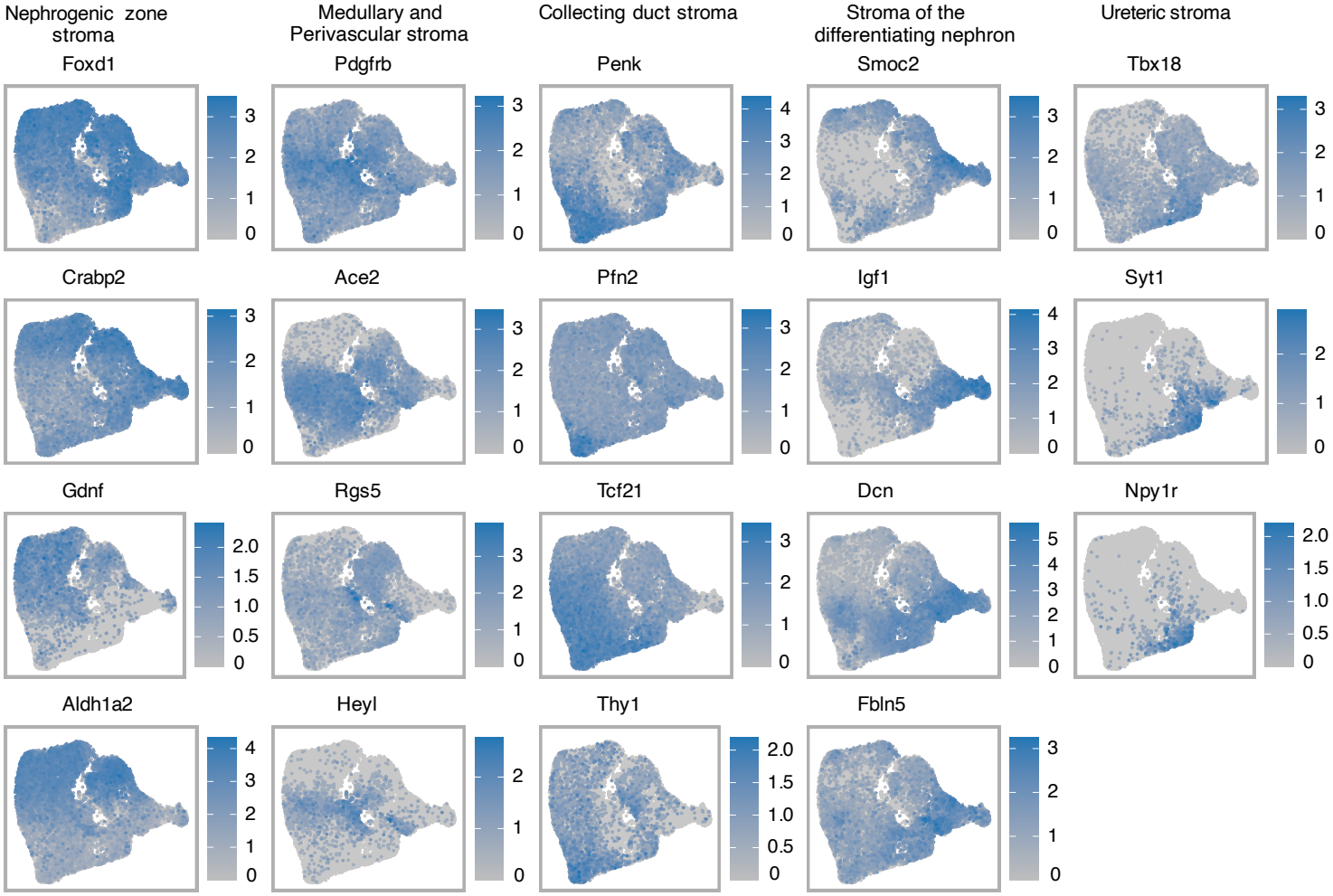
Fig 3

a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Collecting duct stroma
Stroma of the differentiating nephron
Ureteric stroma
Foxd1
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Penk
Smoc2
Tbx18
Crabp2
Ace2
Pfn2
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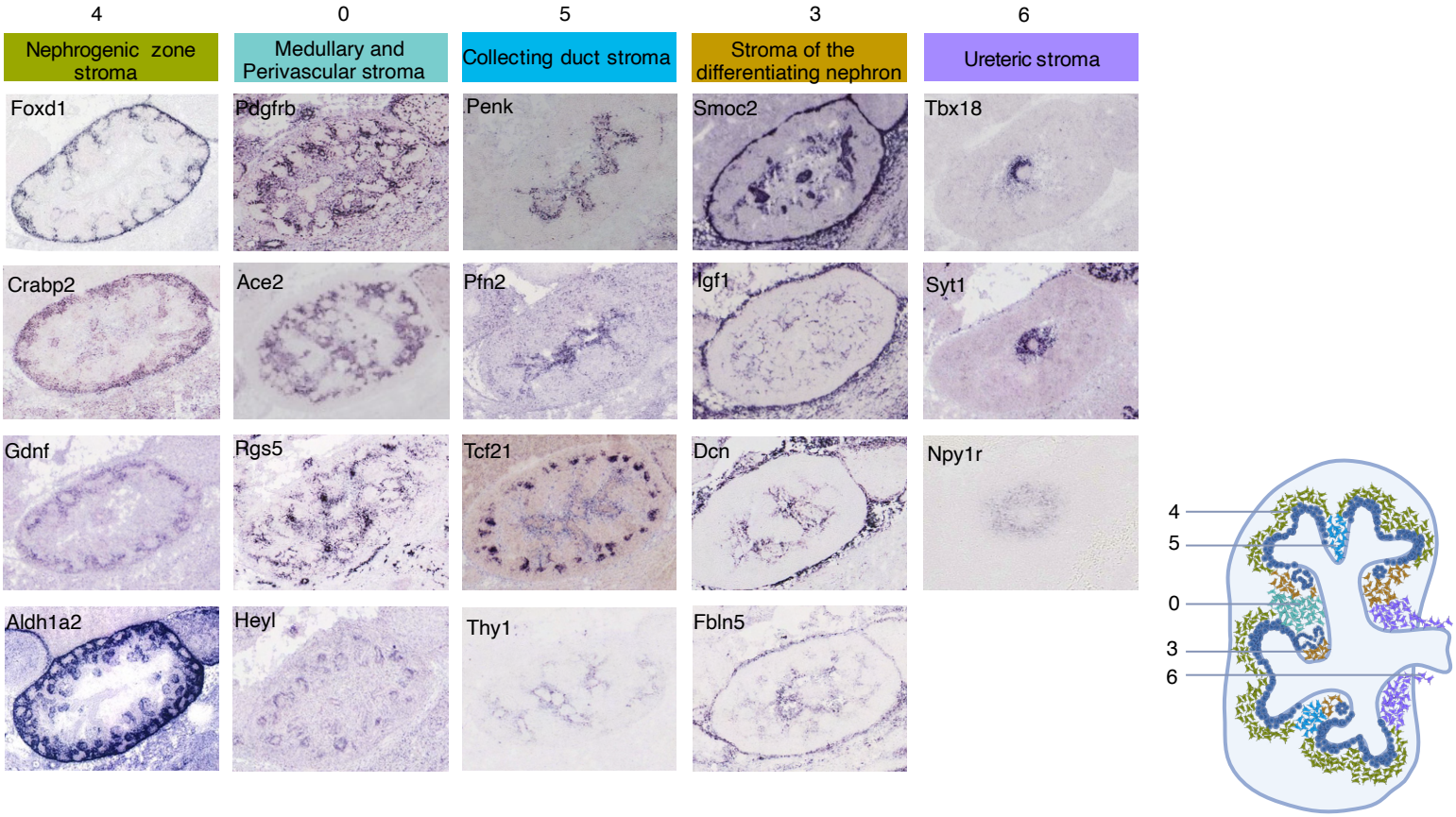
b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Penk
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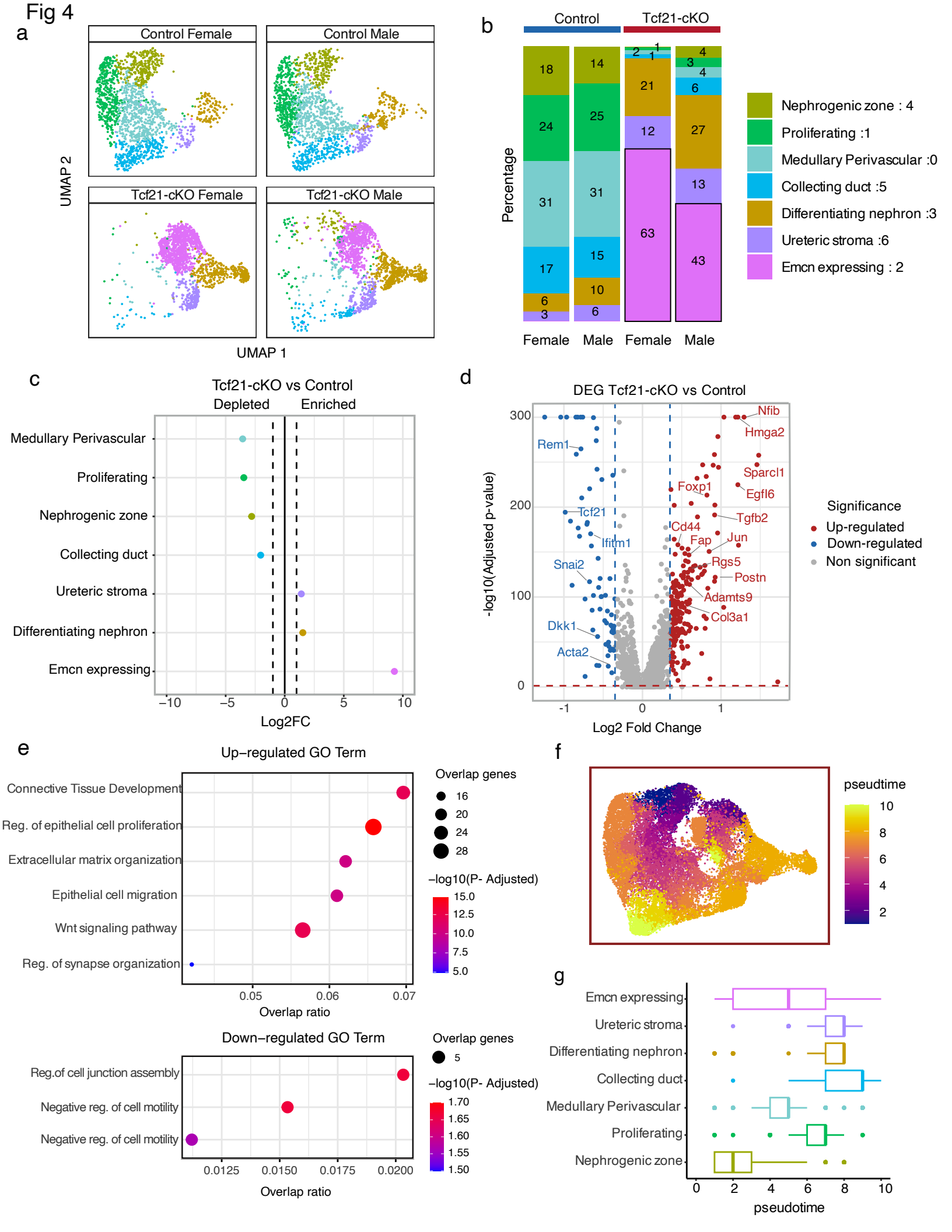
Fig 4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Control Male
Tcf21-cKO Female
Tcf21-cKO Male
UMAP 2
UMAP 1
b
Control
Tcf21-cKO
Percentage
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Male
Female
Male
Nephrogenic zone : 4
Proliferating :1
Medullary Perivascular :0
Collecting duct :5
Differentiating nephron :3
Ureteric stroma :6
Emcn expressing : 2
c
Tcf21-cKO vs Control
Depleted
Enriched
Medullary Perivascular
Proliferating
Nephrogenic zone
Collecting duct
Ureteric stroma
Differentiating nephron
Emcn expressing
Log2FC
d
DEG Tcf21-cKO vs Control
-log10(Adjusted p-value)
Log2 Fold Change
Nfib
Hmga2
Sparcl1
Egfl6
Foxp1
Tgfb2
Cd44
Fap
Jun
Rgs5
Postn
Adamts9
Col3a1
Rem1
Tcf21
Ifitm1
Snai2
Dkk1
Acta2
Significance
Up-regulated
Down-regulated
Non significant
e
Up−regulated GO Term
Connective Tissue Development
Reg. of epithelial cell proliferation
Extracellular matrix organization
Epithelial cell migration
Wnt signaling pathway
Reg. of synapse organization
Overlap ratio
Overlap genes
−log10(P- Adjusted)
Down−regulated GO Term
Reg.of cell junction assembly
Negative reg. of cell motility
Negative reg. of cell motility
Overlap ratio
Overlap genes
−log10(P- Adjusted)
f
pseudtime
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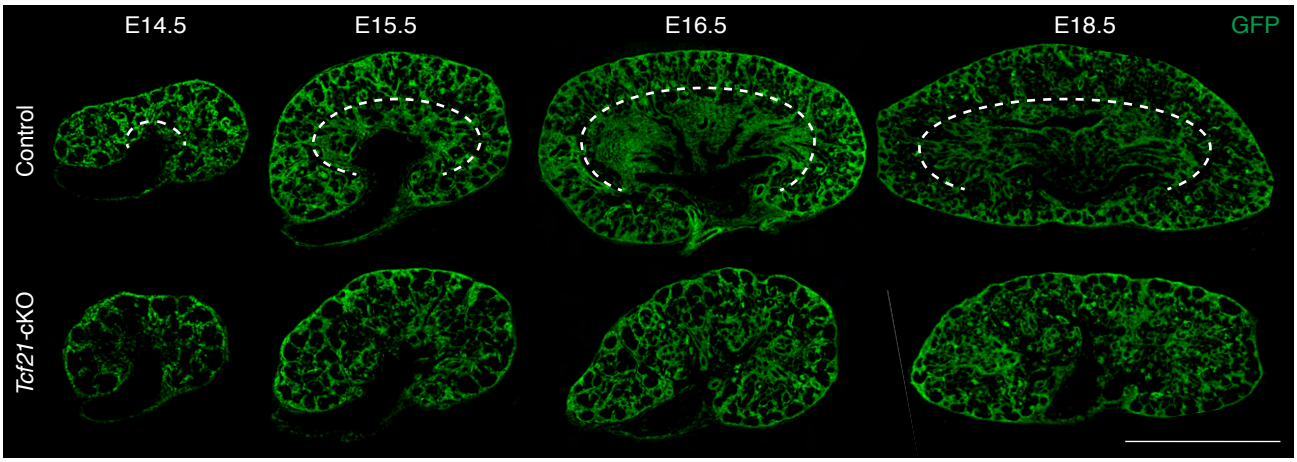
Emcn expressing
Ureteric stroma
Differentiating nephron
Collecting duct
Medullary Perivascular
Proliferating
Nephrogenic zone
pseudotime

Fig 5

a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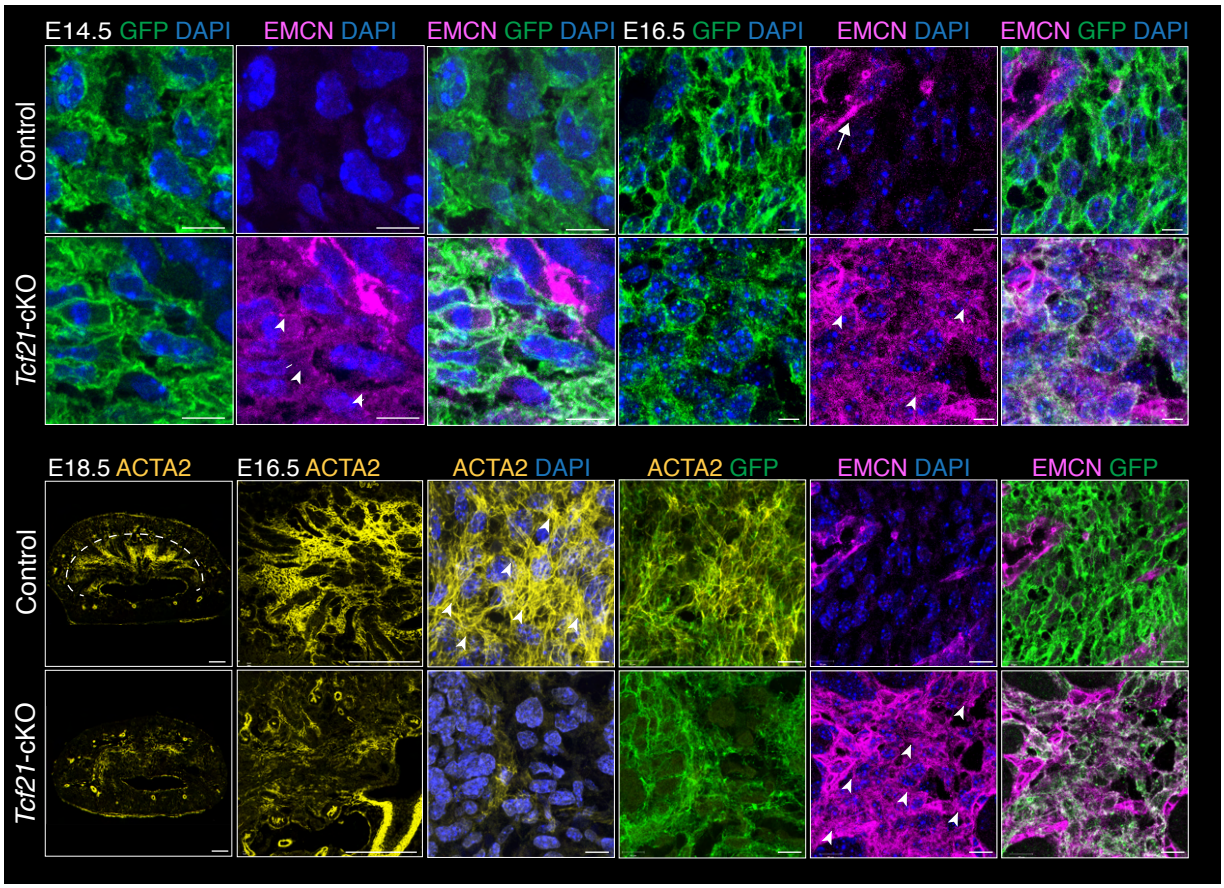
Tcf21-cKO

b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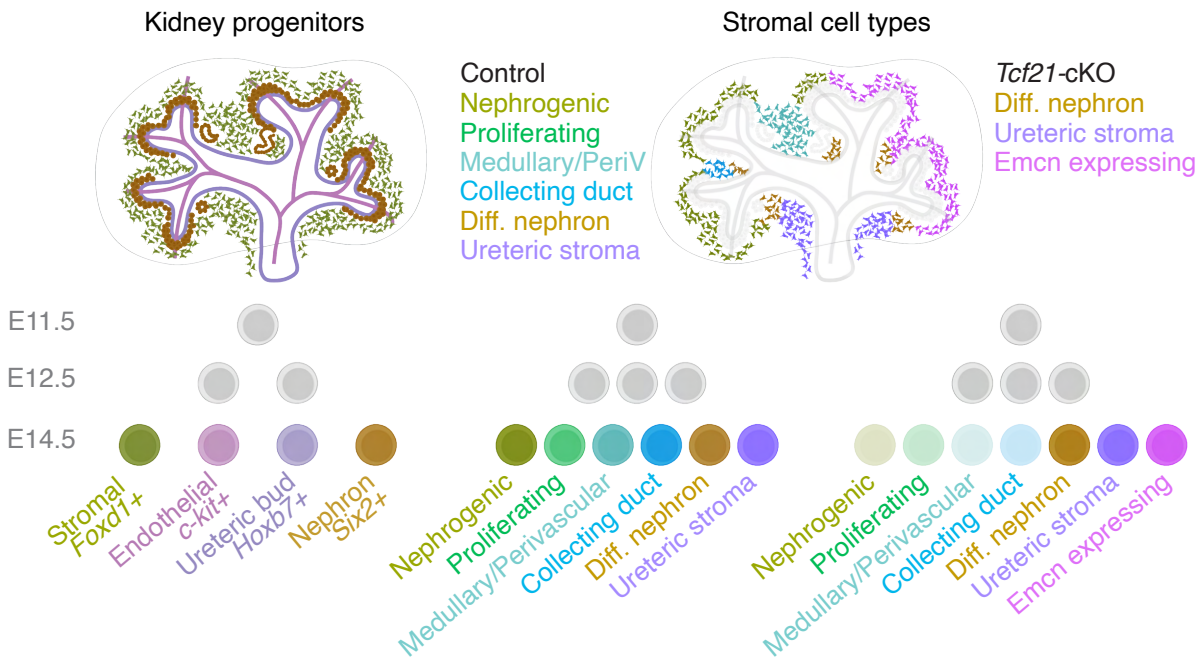
Control
Tcf21-cKO

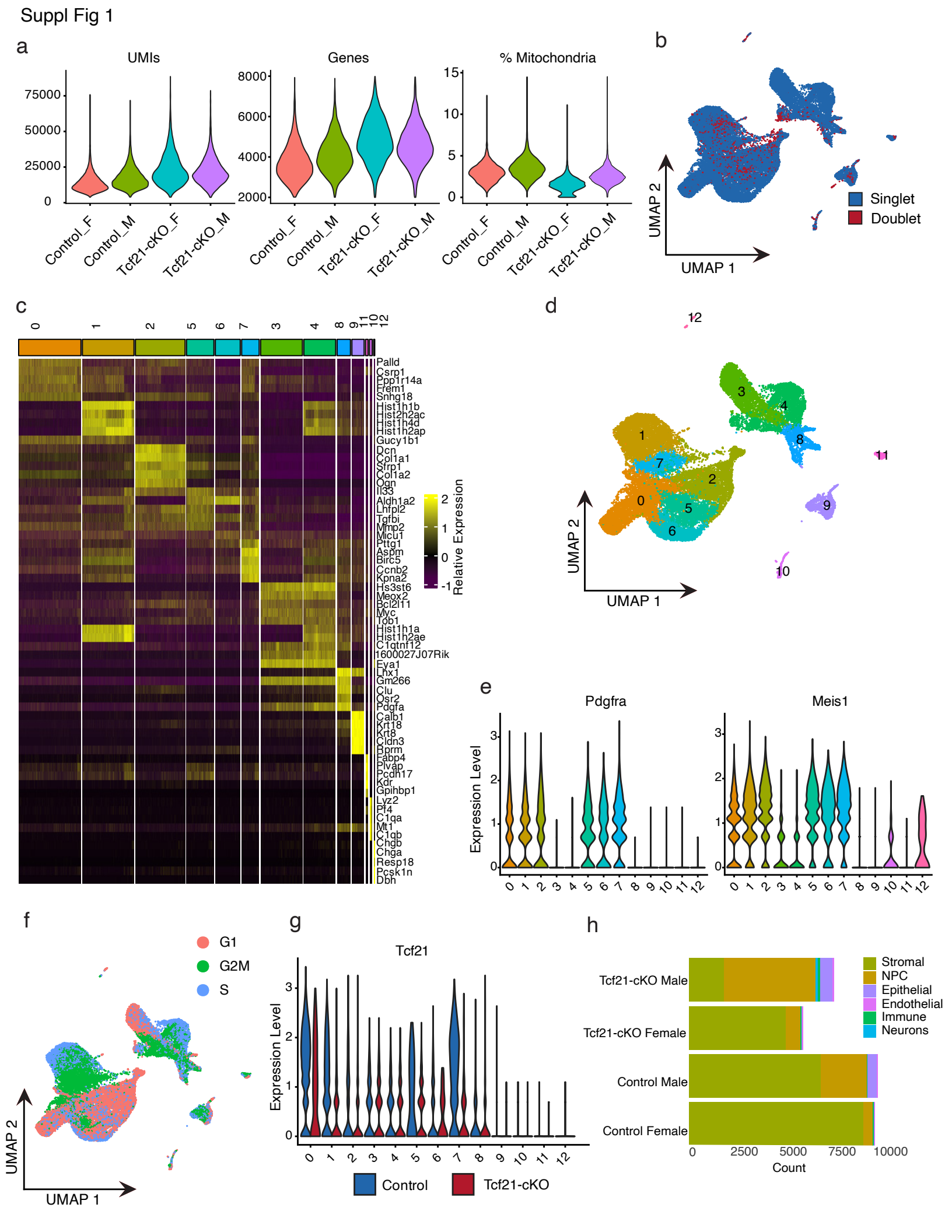
c

E18.5 ACTA2
E16.5 ACTA2
ACTA2 DAPI
ACTA2 GFP
EMCN DAPI
EMCN GFP
Control
Tcf21-cKO

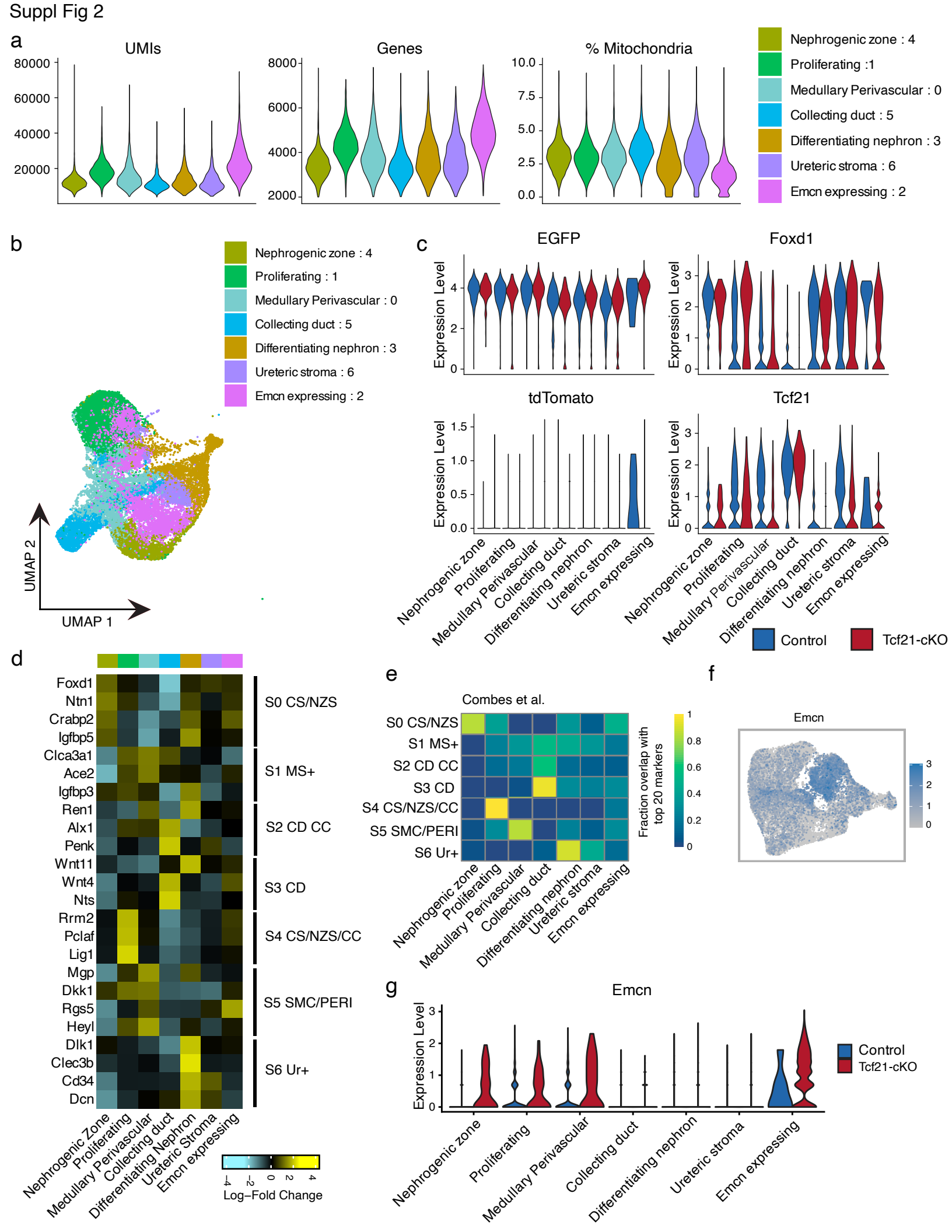
d


Kidney progenitors
Stromal cell types
Control
Nephrogenic
Proliferating
Medullary/PeriV
Collecting duct
Diff. nephron
Ureteric stroma
Tcf21-cKO
Diff. nephron
Ureteric stroma
Emcn expressing
E11.5
E12.5
E14.5
Stromal Foxd1+
Endothelial c-kit+
Ureteric bud Hoxb7+
Nephron Six2+
Nephrogenic
Proliferating
Medullary/Perivascular
Collecting duct
Diff. nephron
Ureteric stroma
Nephrogenic
Proliferating
Medullary/Perivascular
Collecting duct
Diff. nephron
Ureteric stroma
Emcn expressing

Suppl Fig 1


a
UMIs
Genes
% Mitochondria
Control_F
Control_M
Tcf21-cKO_F
Tcf21-cKO_M
b
UMAP 2
UMAP 1
Singlet
Doublet
c
Relative Expression
d
UMAP 2
UMAP 1
e
Pdgfra
Meis1
Expression Level
f
G1
G2M
S
UMAP 2
UMAP 1
g
Tcf21
Expression Level
Control
Tcf21-cKO
h
Tcf21-cKO Male
Tcf21-cKO Female
Control Male
Control Female
Stromal
NPC
Epithelial
Endothelial
Immune
Neurons
Count

Suppl Fig 2


a
UMIs
Genes
% Mitochondria
Nephrogenic zone : 4
Proliferating :1
Medullary Perivascular : 0
Collecting duct : 5
Differentiating nephron : 3
Ureteric stroma : 6
Emcn expressing : 2
b
UMAP 1
UMAP 2
c
EGFP
Foxd1
tdTomato
Tcf21
Expression Level
Control
Tcf21-cKO
d
Foxd1
Ntn1
Crabp2
Igfbp5
Clca3a1
Ace2
Igfbp3
Ren1
Alx1
Penk
Wnt11
Wnt4
Nts
Rrm2
Pclaf
Lig1
Mgp
Dkk1
Rgs5
Heyl
Dlk1
Clec3b
Cd34
Dcn
S0 CS/NZS
S1 MS+
S2 CD CC
S3 CD
S4 CS/NZS/CC
S5 SMC/PERI
S6 Ur+
Log–Fold Change
e
Combes et al.
Fraction overlap with top 20 markers
f
Emcn
g
Emcn

Suppl Fig 3

Cell Type	Marker	Reference
Pan-stroma	Pdgfra	1,2
	Col3a1	1,2
	Meis1	1,2
	Cited1	1,2
	Meis2	1,2,3
Cortical/Nephrogenic Zone	Ntn1	1,3
	Igfbp5	1,3
	Foxd1	1,2,3
	Plag1	3
	Aldh1a2	3
	Gdnf	3
	Smoc2	2
	Fibin	2
Medullary Stroma	Clca3a1	1,3
	Ace2	1,4
	Igfbp3	1,3
	Acta2	1,2,4,5
	Col4a1	3
	Zeb2	3
	Col1a1	3
	Tgfb1i1	2
Perivascular	Akr1b7	2
	Ren1	2
	Rgs5	1,3
	Heyl	1,3
	Pdgfrb	1,3,5
	Igf	1,2
	Mef2c	3,5
	Angpt2	1,3
Collecting Duct Stroma	Wnt4	1,3
	Penk	1,2,3
	Alx1	1,3
	Sema3c	1,4
	Lef1	2,5
	Nkd1	3
	Col15a1	1,3
Ureteric Stroma	Dcn	1,3
	Sparc	1
	Igf1	2,3
	Tbx18	2,3,4,6
	Sfrp2	3
	Dlk1	1,2
	Igbp6	3

Reference:

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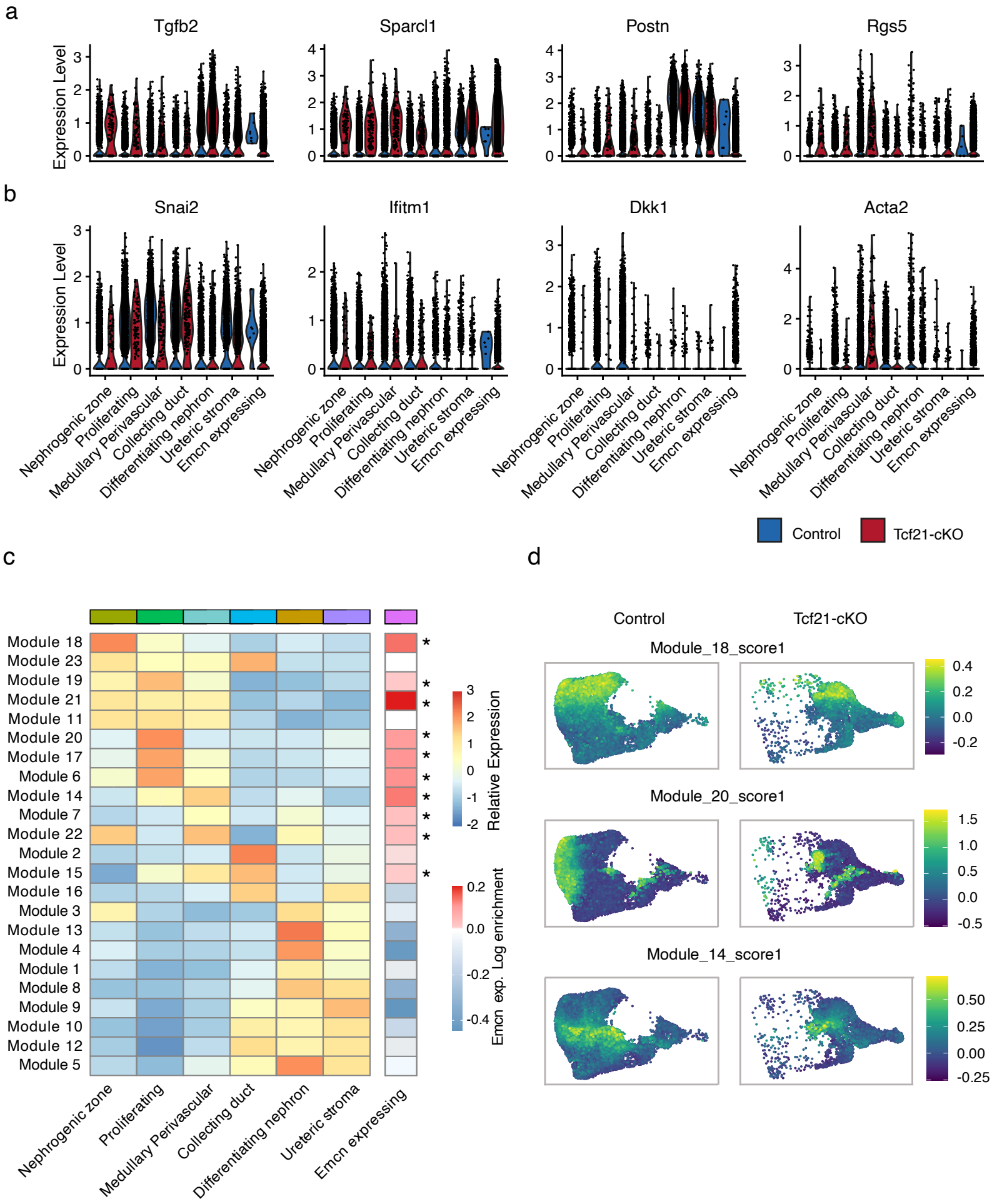
Suppl Fig 4


a
Tgfb2
Sparcl1
Postn
Rgs5
Expression Level
b
Snai2
Ifitm1
Dkk1
Acta2
Expression Level
Nephrogenic zone
Proliferating
Medullary Perivascular
Collecting duct
Differentiating nephron
Ureteric stroma
Emcn expressing
Control
Tcf21-cKO
c
Module 18
Module 23
Module 19
Module 21
Module 11
Module 20
Module 17
Module 6
Module 14
Module 7
Module 22
Module 2
Module 15
Module 16
Module 3
Module 13
Module 4
Module 1
Module 8
Module 9
Module 10
Module 12
Module 5
Relative Expression
Emcn exp. Log enrichment
d
Control
Tcf21-cKO
Module_18_score1
Module_20_score1
Module_14_score1