Supplemental Information

Repression of interstitial identity in nephron progenitor cells by *Pax2* establishes the nephron-interstitium boundary throughout kidney development

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Inventory of Supplemental Information

Supplemental Figures

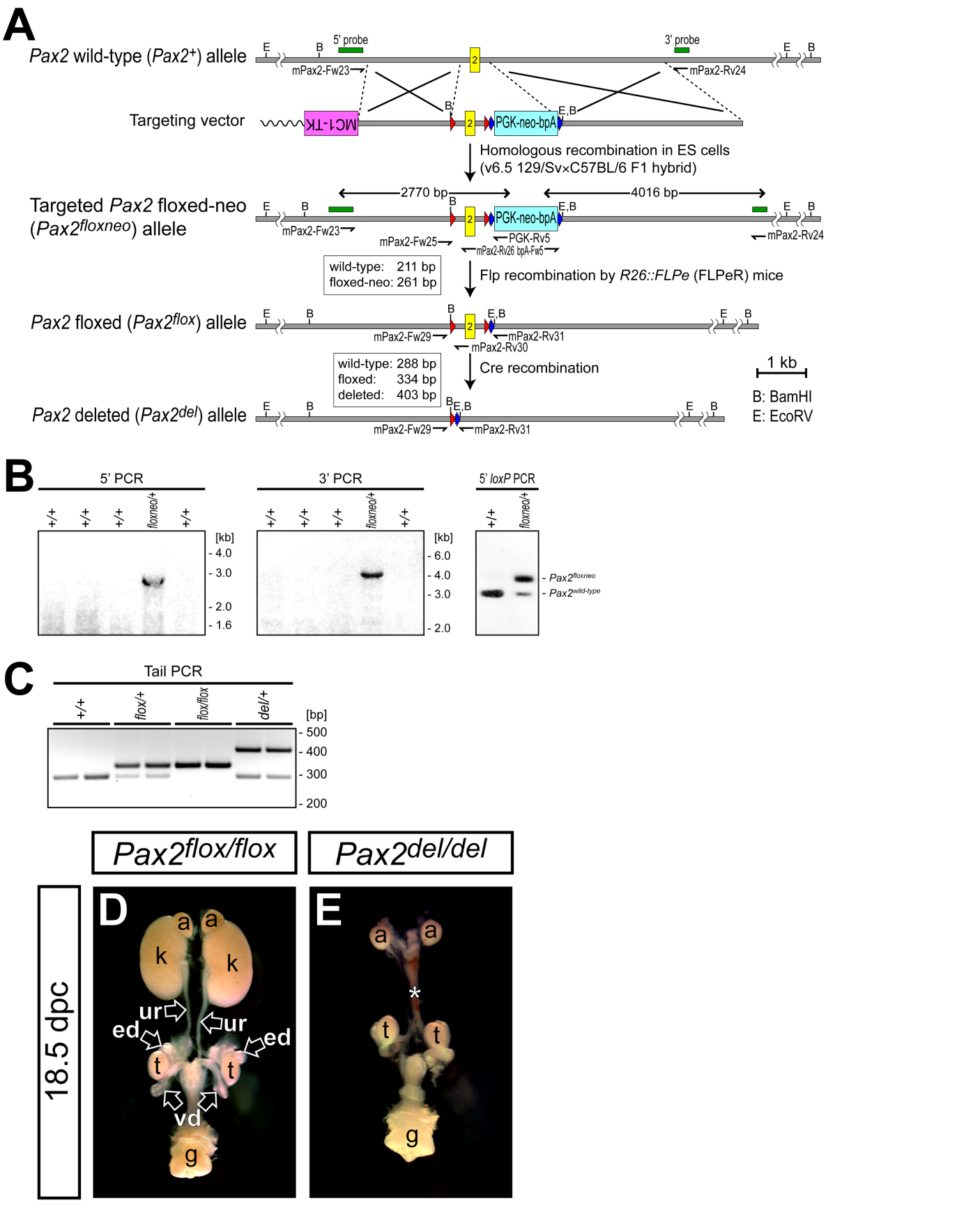

Figure S1. Generation of a Cre-dependent *Pax2*-null allele in the mouse, Related to Figure 1.

(**A**) Schematic illustration for generation of a conditional *Pax2*-null allele. Using homologous recombination in ES cells, two *lox*P sites (red triangles) flanking the exon 2 (yellow box) of the *Pax2* gene were introduced. PGK-neo and MC1-TK were used for positive and negative selection, respectively. After generating mice by blastocyst injection, the FRT (blue triangles)-flanked PGK-neo cassette was removed by breeding with *R26-FLPe* (FLPeR) mice (Farley et al., 2000). Deletion of the exon 2 by Cre recombination causes a frame-shift mutation with a premature translational termination. (**B**) Screening of a correctly targeted ES cell line by 5' PCR (mPax2-Fw23 and PGK-Rv5) and 3' PCR (bpA-Fw5 and mPax2-Rv24), which give 2,770 and 4,016-bp bands for the $Pax2^{floxneo}$ allele, respectively. Because the 5' *loxP* is not linked with the neo selection cassette, PCR screening for the 5' *loxP* site was performed by PCR (mPax2-Fw25 and mPax2-Rv26), which give 211 and 261-bp bands for $Pax2^{+}$ and $Pax2^{floxneo}$ alleles, respectively. (**C**) PCR genotyping (mPax2-Fw29, mPax2-Rv30 and mPax2-Rv31) using genomic DNA from the tail of wild-type, $Pax2^{flox/+}$, $Pax2^{flox/flox}$, and $Pax2^{del/+}$ mice. The $Pax2^{+}$, $Pax2^{flox}$ and $Pax2^{del}$ alleles give 288, 334, 403-bp bands, respectively. (**D and E**) Validation of the conditional *Pax2* null allele. Intercrossing heterozygous $Pax2^{flox/+}$ and $Pax2^{del/+}$ mice generated homozygous $Pax2^{flox/flox}$ and $Pax2^{del/del}$ individuals, respectively. Whole-mount view of urogenital systems at 18.5 dpc from $Pax2^{flox/flox}$ (D) and $Pax2^{del/del}$ (E) males. While $Pax2^{flox/flox}$ mice were phenotypically normal (D), $Pax2^{del/del}$ mice showed phenotypes identical to those in the *Pax2*-null ($Pax2^{-/-}$) mutants previously generated (Torres et al., 1995), including agenesis of the kidney, ureter and reproductive tract such as the epididymis and vas deferens in males (E). The asterisk in E indicates the dorsal aorta, which was removed in D. a, adrenal gland; ed, epididymis; g, genitalia; k, kidney; t, testis; ur, ureter; vd, vas deferens.

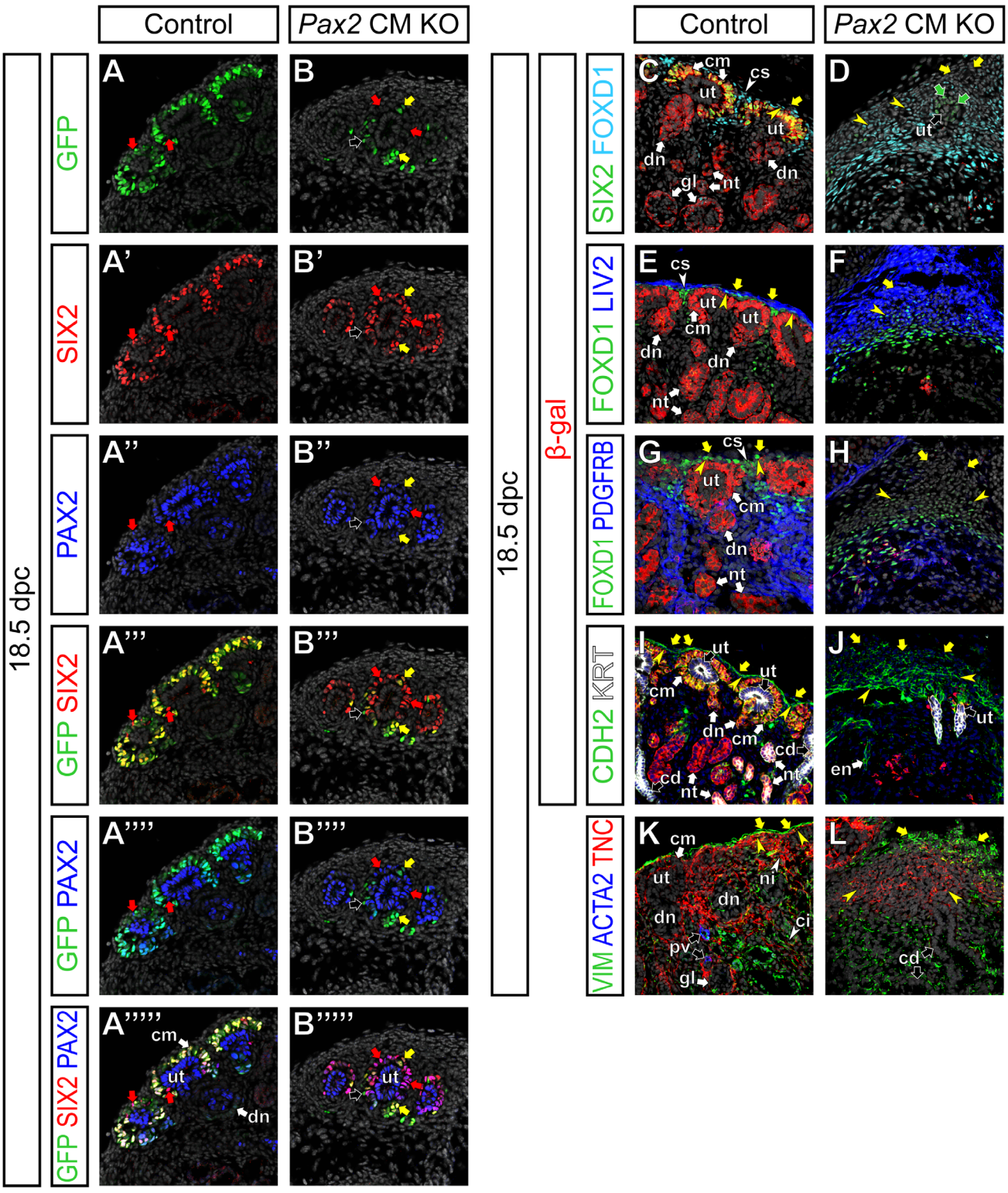

Figure S2. Focal expansion of the renal capsule and connective tissue around rare ureteric tips in cap mesenchyme-specific *Pax2* mutant kidneys, Related to Figure 2.
Confocal immunofluorescence of the cortical region of kidneys from *Six2-eGFPCre*$^{tg/+}$*; Pax2*$^{flox/+}$*; R26R*$^{lacZ/+}$ control mice (Control) and *Six2-eGFPCre*$^{tg/+}$*; Pax2*$^{flox/del}$*; R26R*$^{lacZ/+}$ cap mesenchyme-specific *Pax2* mutant mice (*Pax2* CM KO) at 18.5 dpc with Hoechst (nucleus; gray in A-H,K,L and blue in I,J) staining. **(A-B'''')** *Six2*-GFP (green), SIX2 (red) and PAX2 (blue) staining. Around rare ureteric tips in A-B'''', red arrows indicate *Six2*-GFP^{-} SIX2^{+} PAX2^{+} cells, yellow arrows indicate *Six2*-GFP^{+}

SIX2^{+} PAX2^{low} cells and black arrows indicate *Six2*-GFP^{+} SIX2$^{low\ or\ -}$ PAX2^{-} cells around rare ureteric tips. *Six2*-GFP^{+} SIX2^{+} PAX2^{low} cells suggest recent inactivation of *Pax2* and most likely lose the cap mesenchyme status, becoming *Six2*-GFP^{+} SIX2$^{low\ or\ -}$ PAX2^{-} cells. **(C and D)** β-gal (cap mesenchyme-derived cells; red), SIX2 (green) and FOXD1 (cyan) staining. Green arrows in D indicate rare SIX2^{+} β-gal^{-} cells **(E and F)** β-gal (red), FOXD1 (green) and LIV2 (blue) staining. **(G and H)** β-gal (red), FOXD1 (green) and PDGFRB (blue) staining. **(I and J)** β-gal (red), CDH2 (green) and cytokeratin (KRT, ureteric tip and collecting duct; blue) staining. **(K and L)** Staining with renal interstitial markers; vimentin (VIM; green), α-smooth muscle actin (ACTA2; blue) and tenascin C (TNC; red). Yellow arrowheads in C-L, FOXD1^{+} CDH2^{+} VIM^{-} TNC^{+} SIX2^{-} PDGFRB^{low} LIV2^{+} ACTA2^{-} renal capsule; yellow arrows in C-L, FOXD1^{-} CDH2^{low} VIM^{+} TNC^{-} SIX2^{-} PDGFRB^{low} LIV2^{+} ACTA2^{-} connective tissue. cd, collecting duct; cm, cap mesenchyme; ci, renal cortical interstitium; cs, renal cortical stroma; dn, differentiating nephron; en, endothelium; gl, glomerulus; ni, nephrogenic interstitium; nt, nephron tubule; pv, peri-vasculature; ut, ureteric tip.

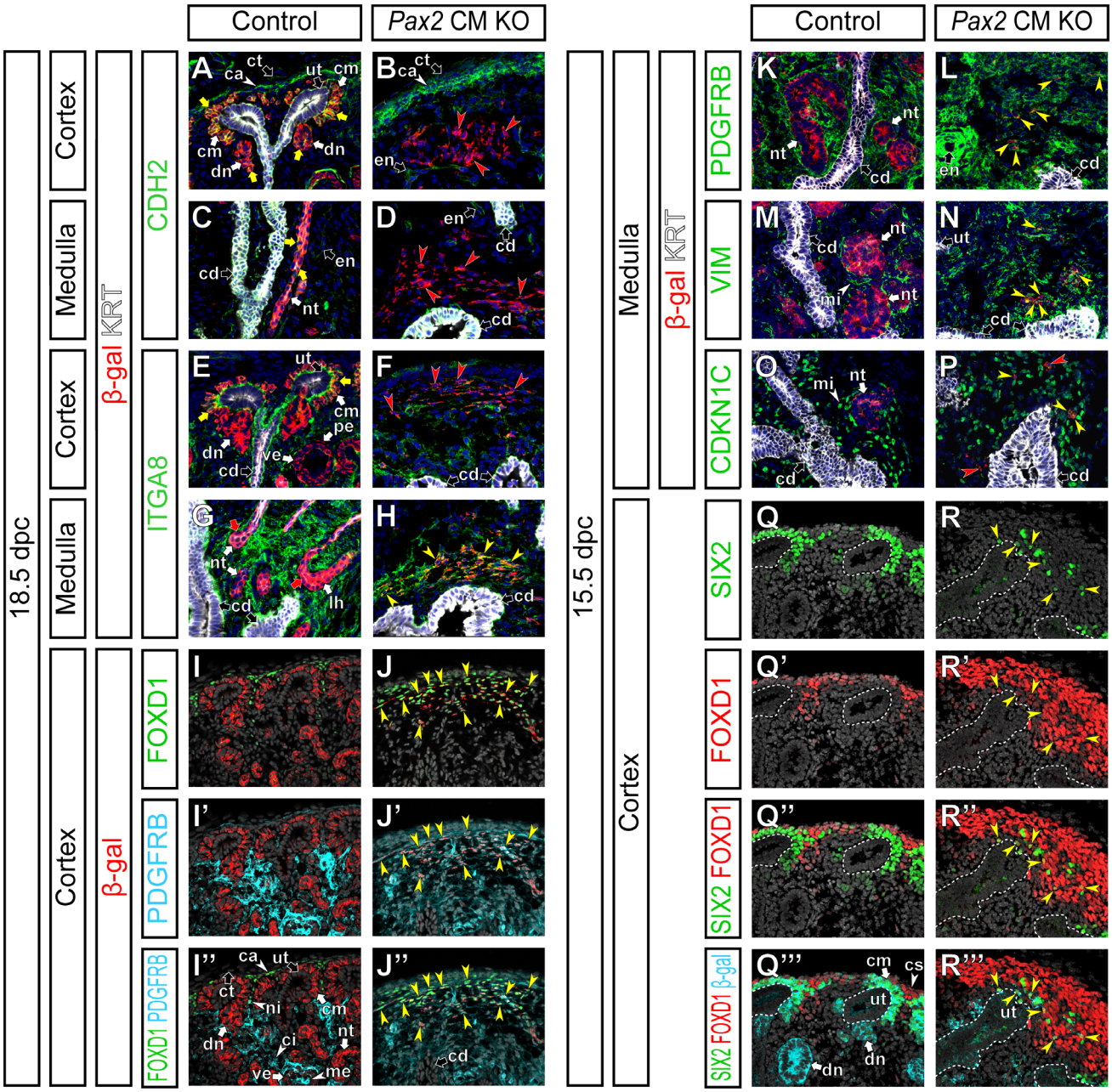

Figure S3. Molecular marker analysis of cap mesenchyme-specific *Pax2* mutant kidneys, Related Figures 4 and 5.

Confocal immunofluorescence of kidneys from Control and *Pax2* CM KO mice. **(A-H)** *Pax2*-deficient cap mesenchyme-derived cells lose gene expression for the nephron lineage, but ectopically gain that for the renal interstitium. The cortical (A,B,E,F) and medullary (C,D,F,H) regions at 18.5 dpc with β-gal (cap mesenchyme-derived cells; red), cytokeratin (KRT, ureteric tip and collecting duct; white) and Hoechst (nucleus; blue) staining. (A-D) CDH2 (N-cadherin; cap mesenchyme, epithelia, endothelium and renal capsule; green) staining. Yellow arrows in A,C, β-gal^{+} CDH2^{+} cells; red arrowheads in B,D, β-gal^{+} CDH2^{-} cells. (E-H) ITGA8 (integrin α8; cap mesenchyme, renal medullary interstitium and collecting duct; green) staining. Yellow arrows in E, β-gal^{+} ITGA8^{+} cap mesenchyme cells in controls; red arrowheads in F, β-gal^{+} ITGA8$^{-\text{ or low}}$ renal interstitial cells in the cortical region of mutants; red arrows in G, β-gal^{+} ITGA8^{-} nephron tubule cells in controls; yellow arrowheads in H, β-gal^{+} ITGA8^{+} renal interstitial cells in the medullary region of mutants. **(I-J")** FOXD1-expressing *Pax2*-deficient cap mesenchyme-derived cells co-express PDGFRB. The cortical region at 18.5 dpc with cytoplasmic β-gal (cap mesenchyme-derived cells; red), nuclear FOXD1 (green), membranous PDGFRB (cyan) and

Hoechst (nucleus; gray) staining. Yellow arrowheads, β-gal^{+} FOXD1^{+} PDGFRB^{+} cells. **(K-P)** *Pax2*-deficient cap mesenchyme-derived cells ectopically express molecular markers for the renal medullary interstitium at 15.5 dpc. The medullary region with cytoplasmic β-gal (cap mesenchyme-derived cells; red), cytokeratin (KRT; collecting duct; white) and Hoechst (nucleus; blue) staining. (K and L) PDGFRB (green) staining in the membrane. Yellow arrowheads in L indicate β-gal^{+} PDGFRB $^{+}$ cells. (M and N) VIM (Vimentin; green) staining in the cytoplasm. Yellow arrowheads in N indicate β-gal^{+} VIM^{+} cells. (O and P) CDKN1C (P57KIP2; green) staining in the nucleus. Yellow and red arrowheads in P indicate β-gal^{+} CDKN1C^{+} and β-gal^{+} CDKN1C^{-} cells, respectively. Note that CDKN1C is expressed in a subset of renal medullary interstitial cells in O and P. **(Q-R''')** *Pax2*-deficient cap mesenchyme-derived cells can express both SIX2 and FOXD1 at 15.5 dpc. The cortical region at 15.5 dpc with β-gal (cap mesenchyme-derived cells; red), SIX2 (green), FOXD1 (red) and Hoechst (nucleus; gray) staining. Yellow arrowheads indicate SIX2^{+} FOXD1^{+} β-gal^{+} cells. White dashed lines indicate the ureteric tip/collecting duct. ca, renal capsule; cd, collecting duct; ci, renal cortical interstitium; cm, cap mesenchyme; cs, renal cortical stroma; ct, connective tissue; dn, differentiating nephron; en, endothelium; lh, loop of Henle; me, mesangium; mi, renal medullary interstitium; ni, nephrogenic interstitium; nt, nephron tubule; pe, parietal epithelium; sb, S-shaped body; ut, ureteric tip, ve, visceral epithelium (podocytes).

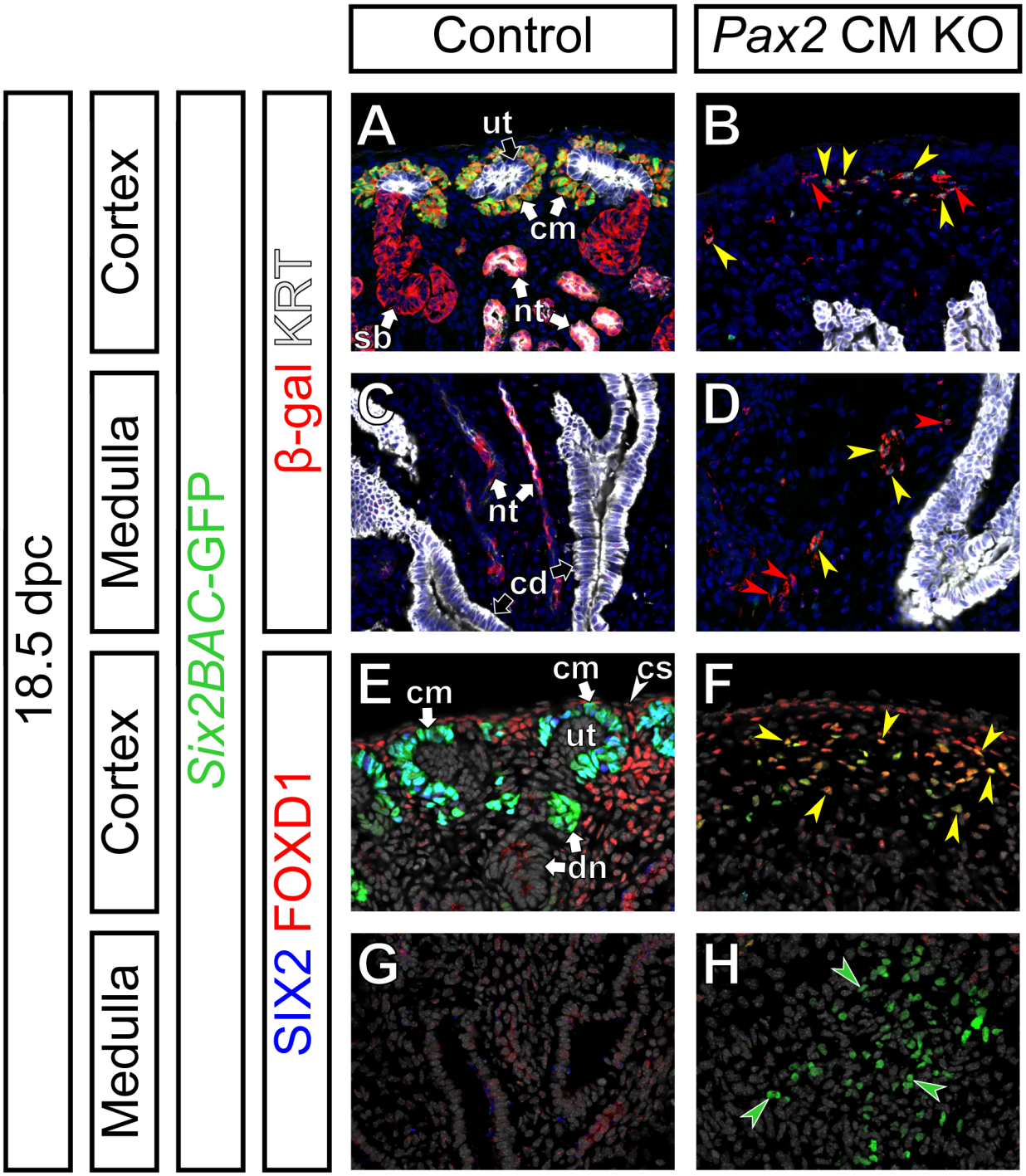

Figure S4. Some renal interstitial cells showed weak GFP expression in cap mesenchyme-specific *Pax2* mutant kidneys, Related Figures 4 and 5.
Confocal immunofluorescence of the cortical (A,B,E,F) and medullary (C,D,G,H) regions of kidneys from Control and *Pax2* CM KO mice at 18.5 dpc with GFP (green) and Hoechst (nucleus; blue in A-D and grey in E-H) staining. **(A-D)** β-gal (red) and cytokeratin (KRT; ureteric tip and collecting duct; white) staining. Yellow and red arrowheads in B and D indicate β-gal^{+} GFP^{+} and β-gal^{+} GFP^{-} cells in *Pax2* CM mutants, respectively. **(E-H)** SIX2 (blue) and FOXD1 (red) staining. Yellow arrowheads in F, GFP^{+} FOXD1^{+} SIX2^{-} cells; green arrowheads in H, GFP^{+} FOXD1^{-} SIX2^{-} cells. cd, collecting duct; cm, cap mesenchyme; cs, renal cortical stroma; dn, differentiating nephron; nt, nephron tubule; sb, S-shaped body; ut, ureteric tip.

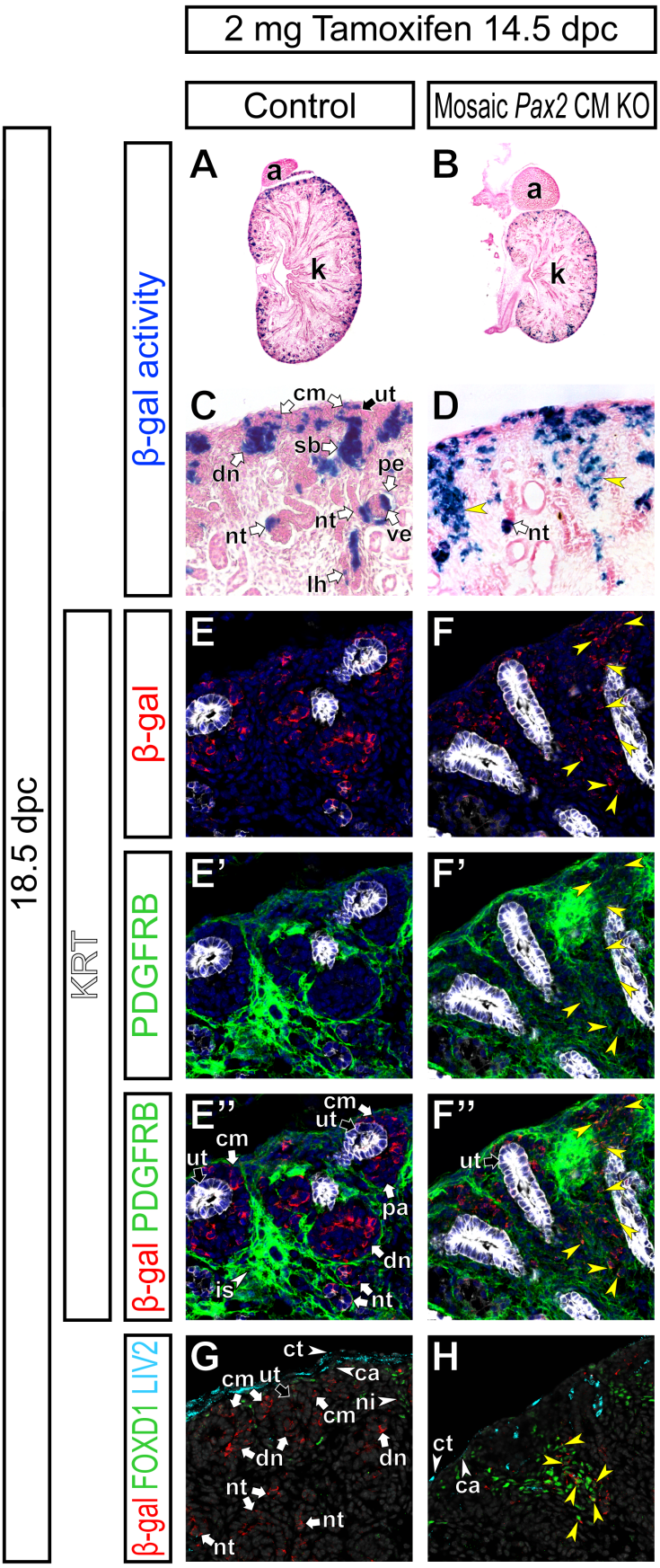

Figure S5. ***Pax2*** **function in cap mesenchyme cells is required cell-autonomously to repress transdifferentiation into renal interstitial cells at 14.5 dpc, Related Figures 6.**

Kidneys at 18.5 dpc from *Six2*$^{eGFPCreERT2/+}$*; Pax2*$^{flox/+}$*; R26R*$^{lacZ/+}$ control mice (Control) and *Six2*$^{eGFPCreERT2/+}$*; Pax2*$^{flox/del}$*; R26R*$^{lacZ/+}$ mosaic cap mesenchyme-specific *Pax2* mutant mice (Mosaic *Pax2* CM KO) after injection of 2 mg of tamoxifen into dams at 14.5 dpc. **(A-D)** X-gal (blue) stained section counter-stained with eosin (pink). C and D show high magnification of cortical regions in A and B, respectively. Yellow arrowheads in D indicate X-gal stained cap mesenchyme-derived cells in the renal interstitium. **(E-F")** Confocal immunofluorescence of the cortical region with β-gal (cap mesenchyme-derived cells; red), PDGFRB (green), cytokeratin (KRT; ureteric tip and collecting duct; white) and Hoechst (nucleus; blue) staining. Yellow arrowheads indicate β-gal$^+$ PDGFRB$^+$ cells. **(G and H)** Confocal immunofluorescence of the cortical region with β-gal (cap mesenchyme-derived cells; red), FOXD1 (green), LIV2 (cyan) and Hoechst (nucleus; gray) staining. Yellow arrowheads in H indicate β-gal$^+$ FOXD1$^+$ cells. a. adrenal gland; ca, renal capsule; cm, cap mesenchyme; ct, connective tissue; dn, differentiating nephron; is, renal interstitium; k, kidney; ni, nephrogenic interstitium; nt, nephron tubule; pa, pretubular aggregate; ut, ureteric tip.

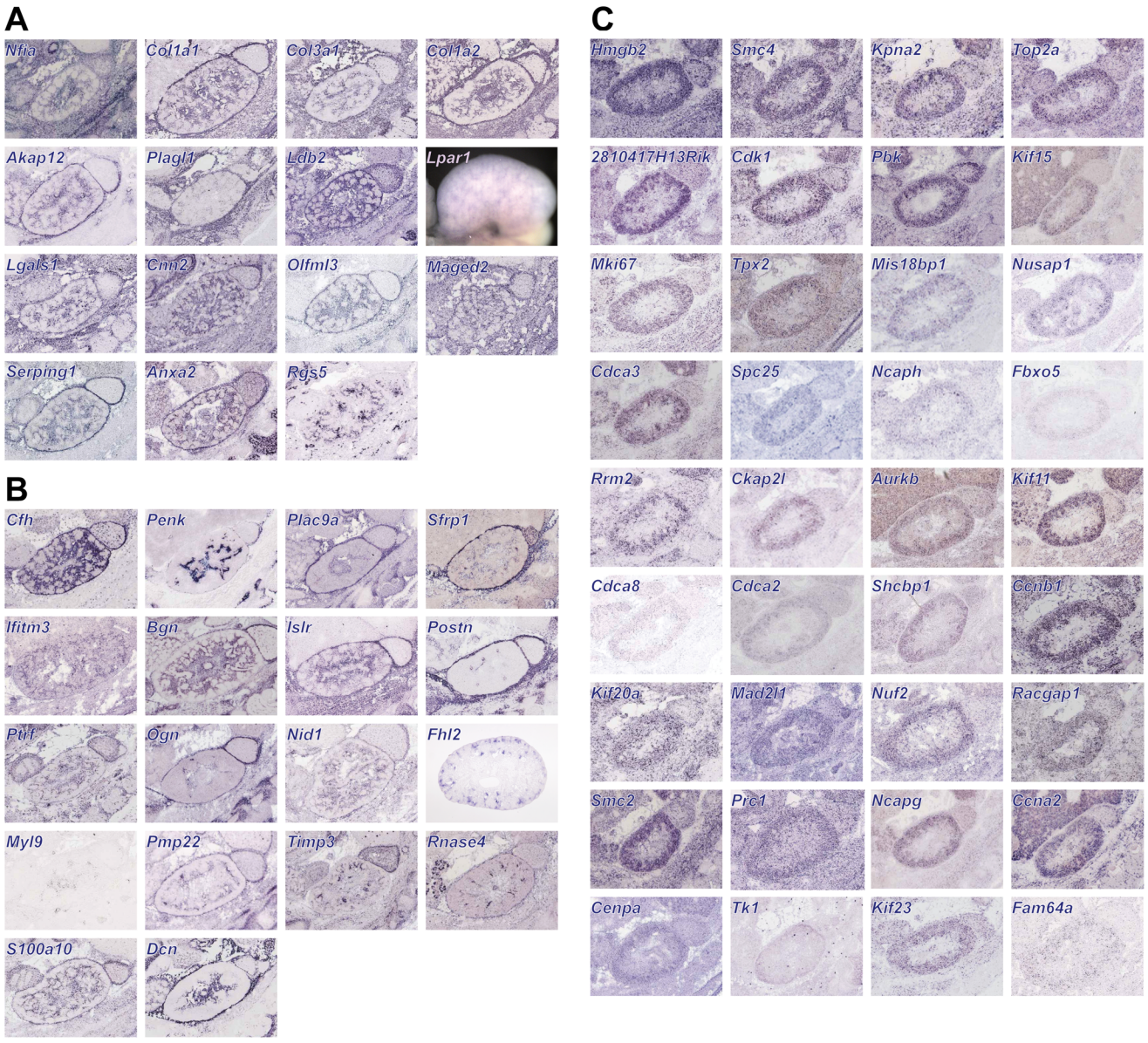

Figure S6. Expression patterns of genes regulated in *Pax2*-mutant cap mesenchyme-derived cells, Related to Figure 7.
Section in situ hybridization at 14.5 dpc from the GenePaint and Euroexpress databases for up- and down-regulated genes in *Pax2*-deficient cap mesenchyme-derived cells, except for *Lpar1* by whole-mount in situ hybridization at 15.5 dpc and *Fhl2* by section in situ at 15.5 dpc from the GUDMAP database. (**A and B**) Genes up-regulated at 13.5 dpc (A) and 18.5 dpc (B). Most genes up-regulated in *Pax2*-mutant cap mesenchyme-derived cells are renal interstitium-specific genes. (**C**) Down-regulated genes. Most genes down-regulated in *Pax2*-mutant cap mesenchyme-derived cells encode cell cycle-regulating genes highly expressed in the nephrogenic zone.

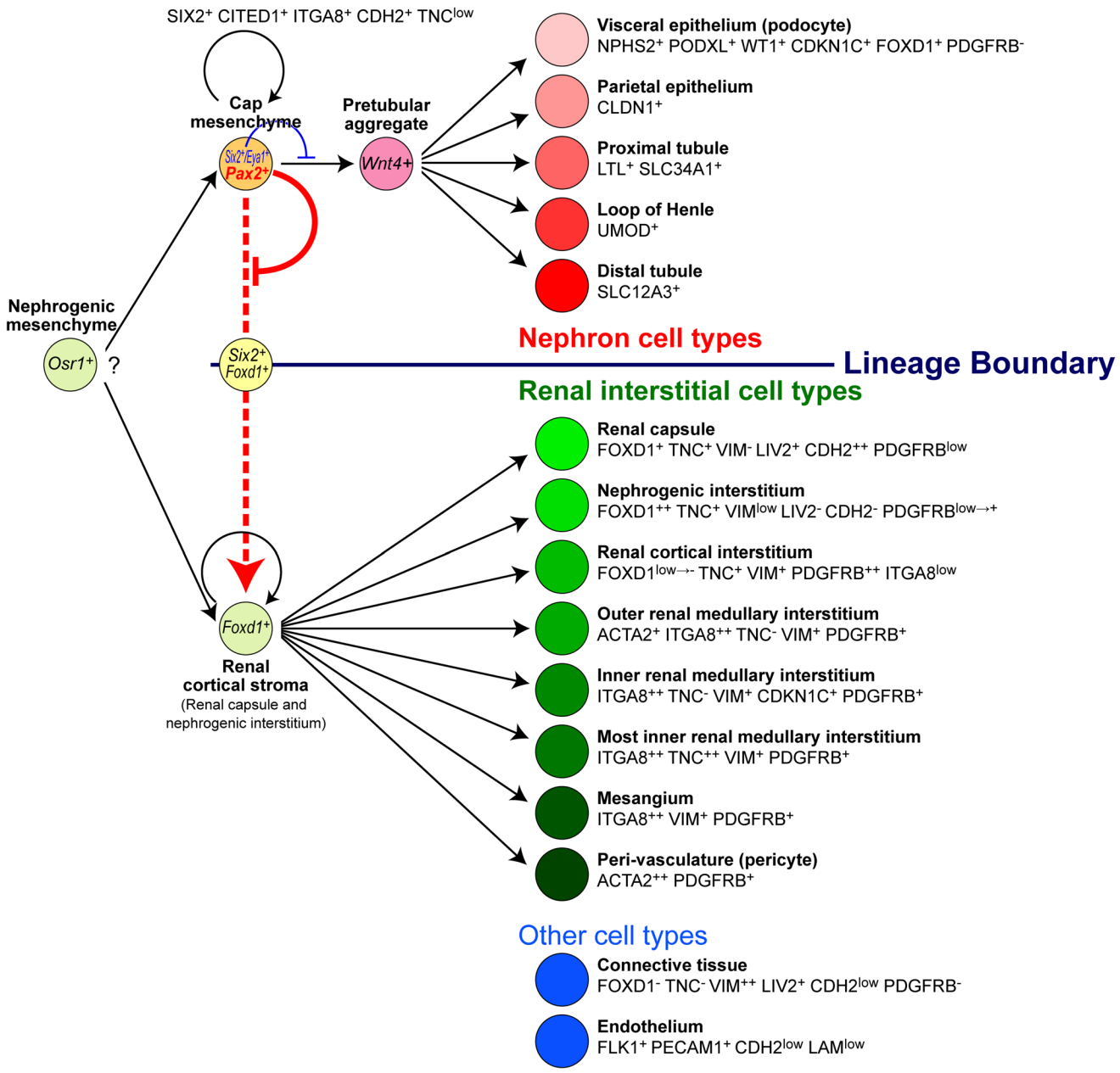

Figure S7. A model for cell type specification in the metanephric mesenchyme during kidney development, Related Figures 4-7.

We previously showed that the *Six2*+ cap mesenchyme and *Foxd1*+ renal cortical stroma are multipotent self-renewing progenitor populations for the nephron epithelia and renal interstitium, respectively (Kobayashi et al., 2014; Kobayashi et al., 2008). There is a lineage boundary between the nephron epithelia and renal interstitium compartments. *Six2* cell-autonomously maintains cap mesenchyme cells by repressing precocious differentiation into differentiating nephron epithelial cells (Kobayashi et al., 2008; Self et al., 2006) cooperating with *Eya1* (Xu et al., 2014). Our observation in this study showed that *Pax2*-deficient cap mesenchyme cells transiently become $SIX2^+$ $FOXD1^+$ double positive cells and subsequently transdifferentiate into renal interstitial cell types. Thus, the lineage boundary between the nephron and renal interstitial compartments is established by the cell-autonomous *Pax2* function in nephron progenitor cells by repressing transdifferentiation into renal interstitial cell fates during kidney development.