SUPPLEMENTAL MATERIAL

Pax proteins mediate segment-specific functions in proximal tubule survival and response to ischemic injury

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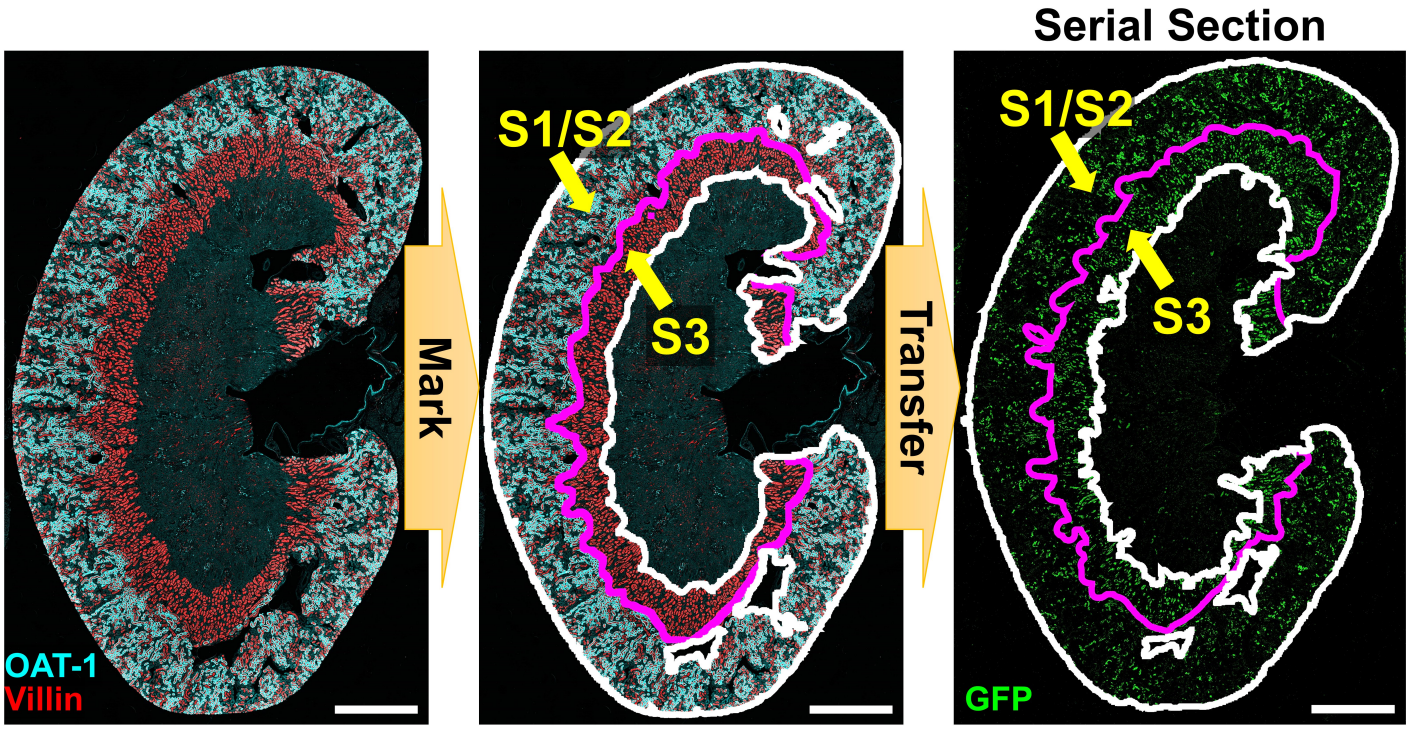
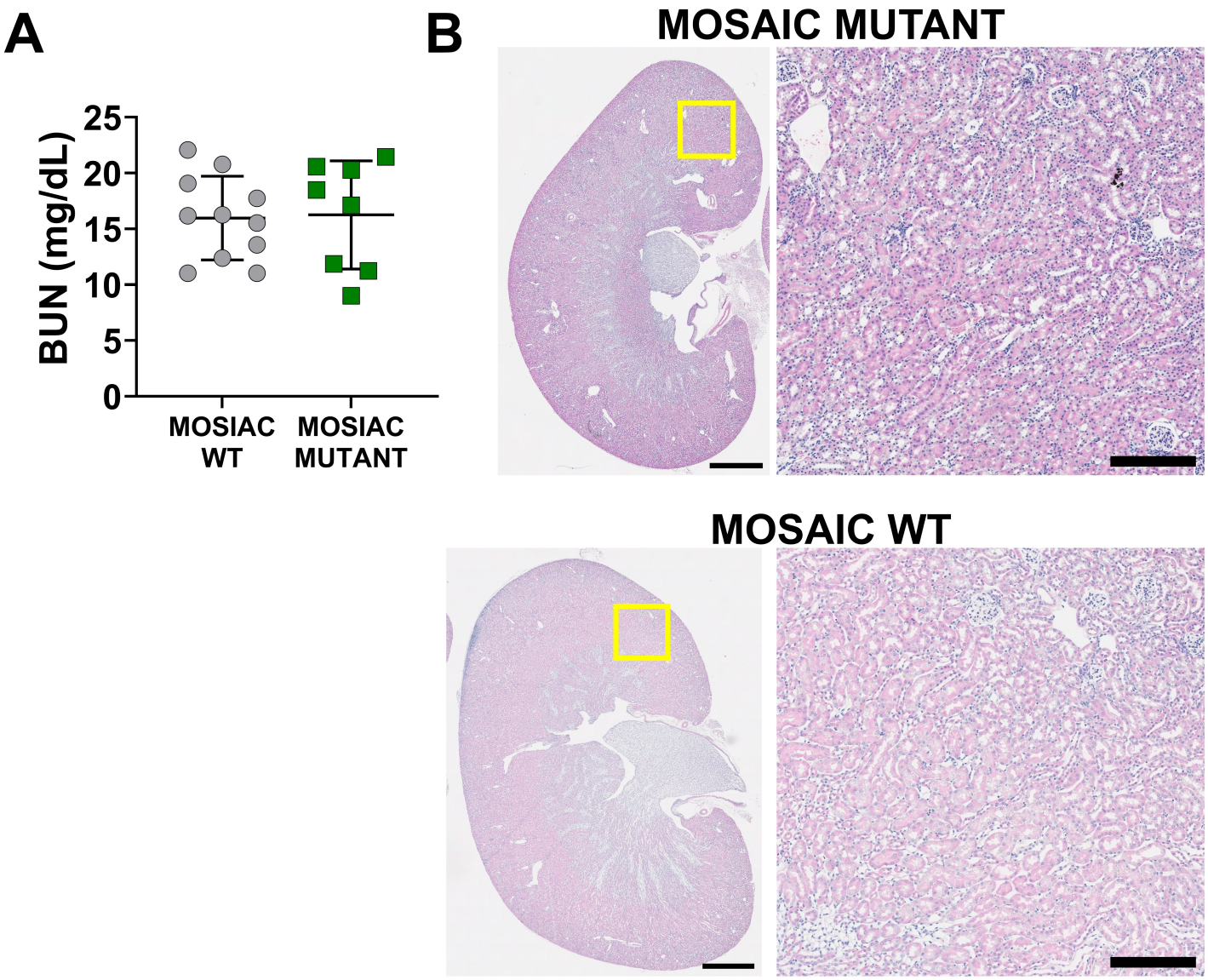
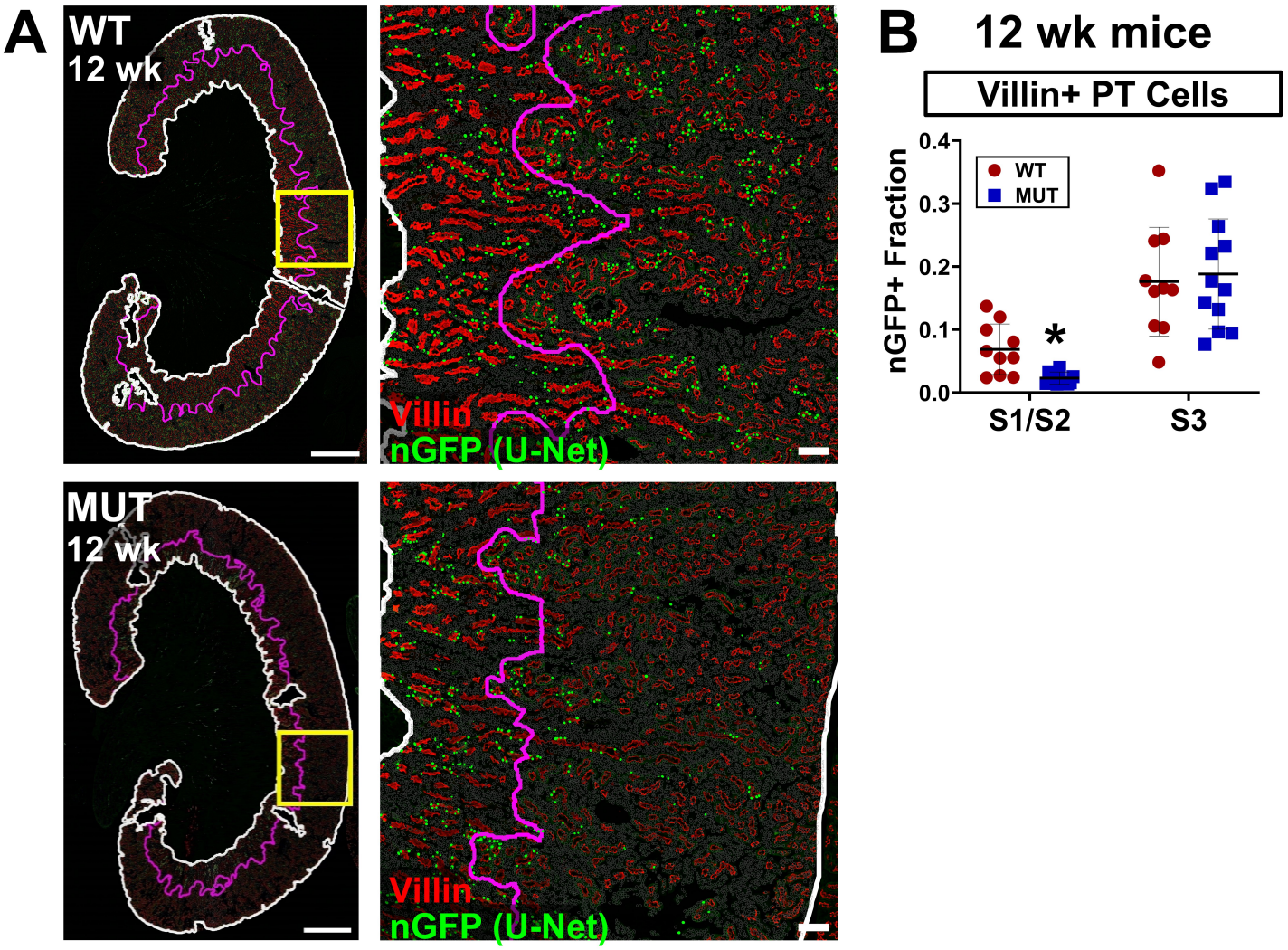
SUPPLEMENTAL FIGURES

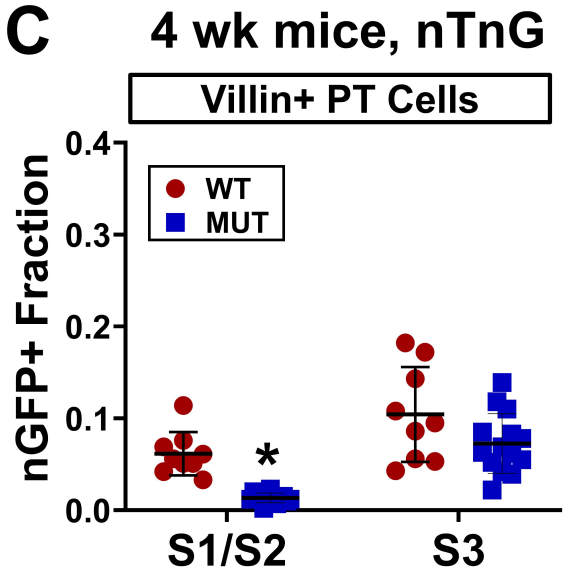


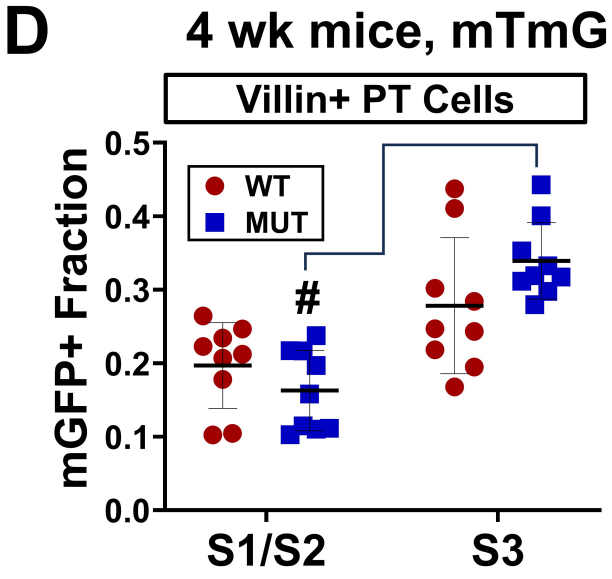
Supplemental Fig. S1. Methodology for distinguishing S1/S2 and S3 proximal tubule. Each batch of sections was stained for organic anion transporter 1 (OAT-1), which marks predominantly S2 proximal tubule and Villin which marks the entire proximal tubule. These allowed for ready identification of the S3 proximal tubule in the outer strip of outer medulla. Proximal tubule outside this region in the cortex was presumed to be predominantly S1 or S2 proximal tubule. This demarcation was transferred to serial sections for subsequent stains using morphological landmarks. Scale bar: 1 mm.



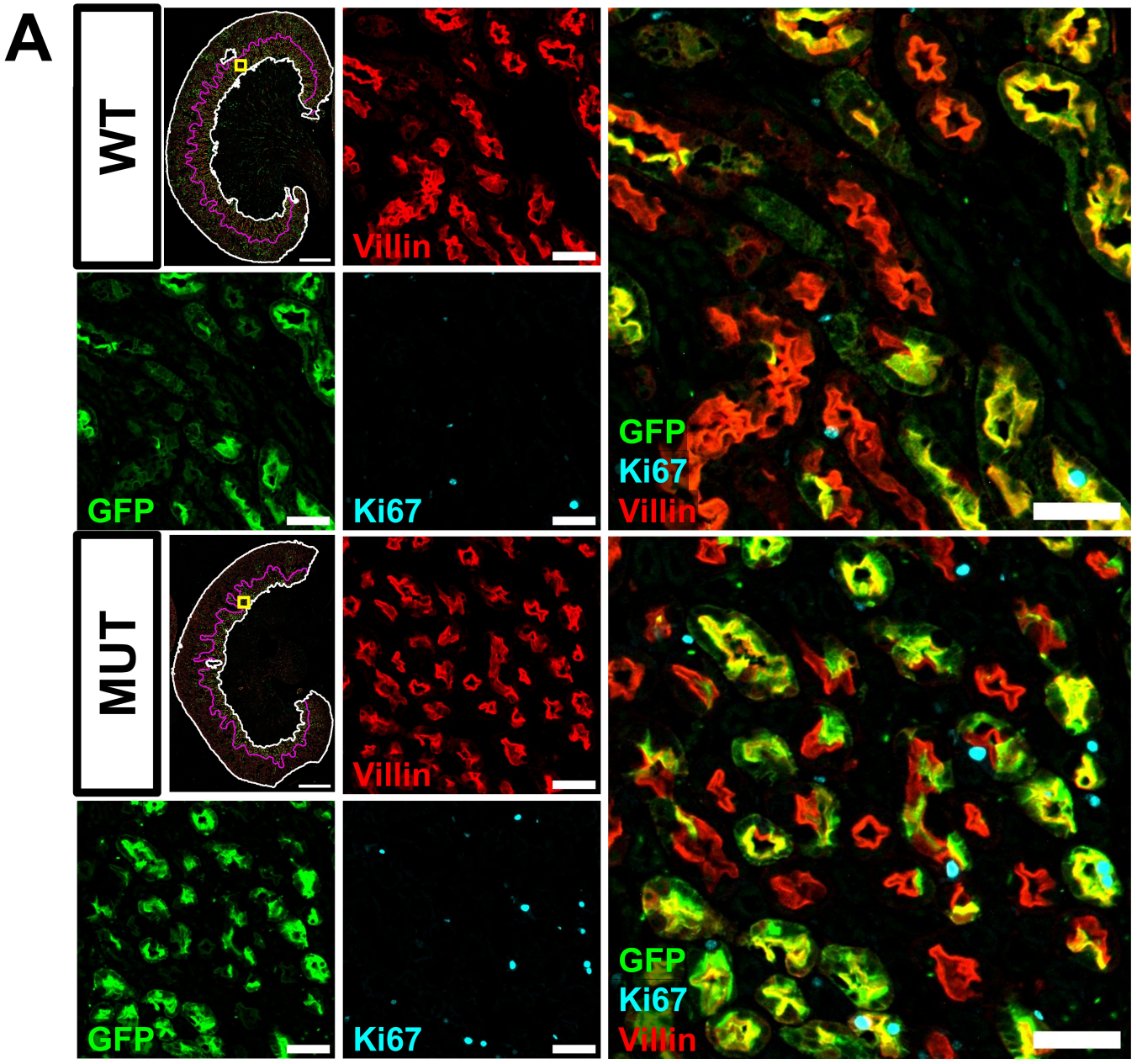
Supplemental Fig. S2. Mosaic Pax2/8 mutants and mosaic wild type (WT) mice have similar kidney function and appearance at baseline. *A*: Blood urea nitrogen (BUN) measured in uninjured 12-wk-old mosaic mutant and mosaic WT mice. *B*: Hematoxylin and eosin stained sections of kidney from 12-wk-old mice showed normal morphology in both strains. Scale bars: whole kidney, 1 mm; inset image, 200 μm.

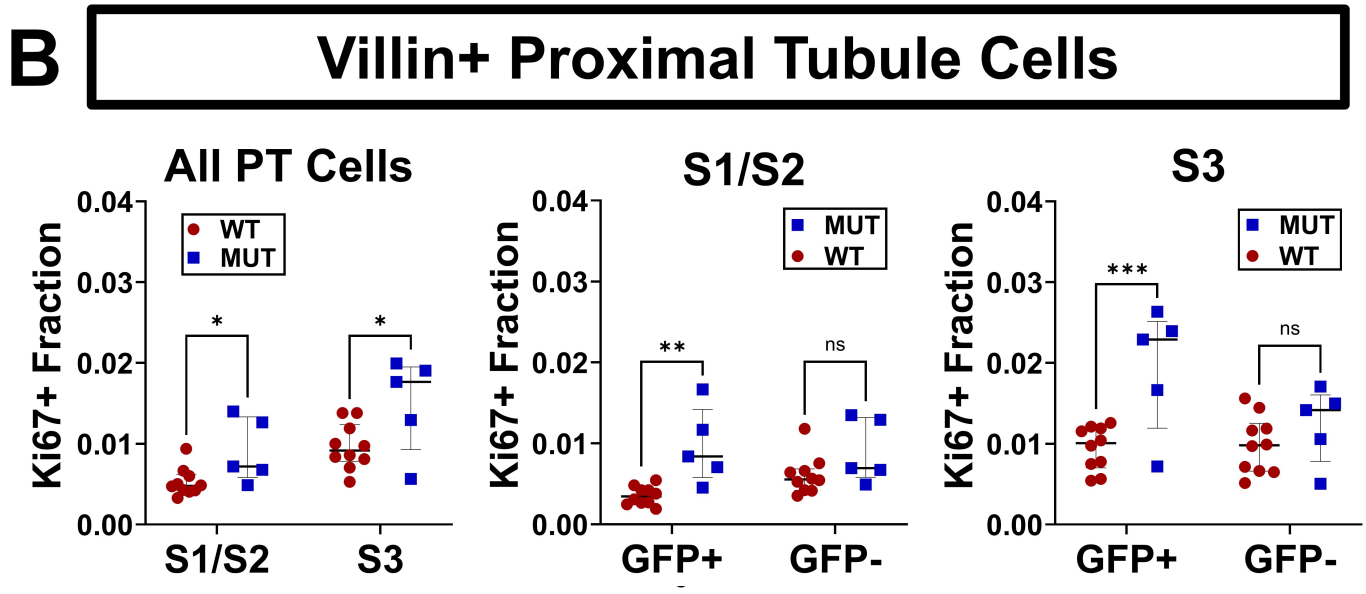

A
WT
12 wk
Villin
nGFP (U-Net)
MUT
12 wk
Villin
nGFP (U-Net)
B
12 wk mice
Villin+ PT Cells
nGFP+ Fraction
WT
MUT
*
S1/S2
S3


C
4 wk mice, nTnG
Villin+ PT Cells
nGFP+ Fraction
WT
MUT
*
S1/S2
S3

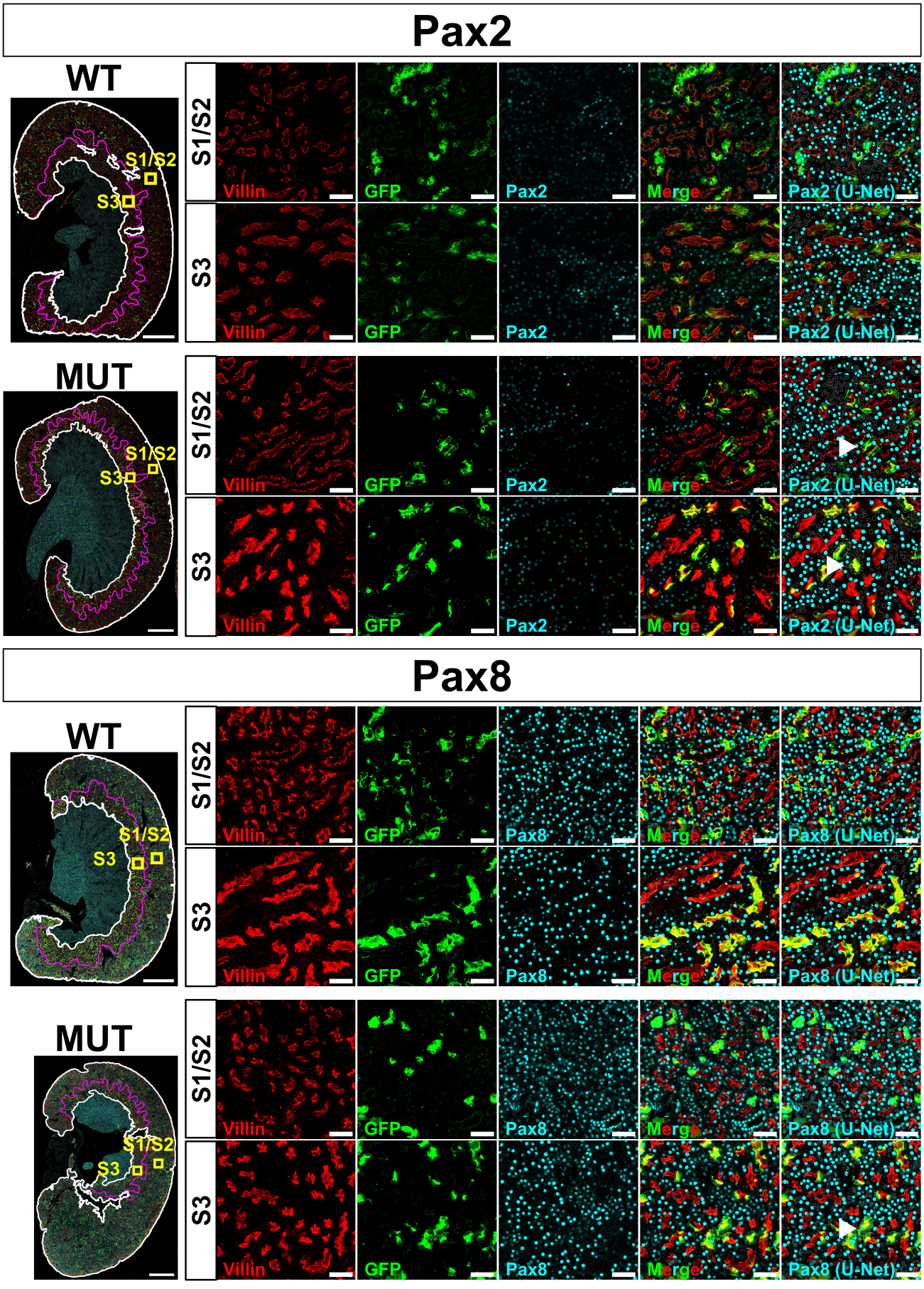

D
4 wk mice, mTmG
Villin+ PT Cells
mGFP+ Fraction
WT
MUT
#
S1/S2
S3

Supplemental Fig. S3: Independent, nuclear reporter mosaic mutant (MUT) mice are depleted of mutant cells in the S1/S2 proximal tubule (PT). *A*: Representative images of the cortex and outer stripe of the outer medulla (OSOM, white outline) of 12-wk-old mosaic wild type (WT) and mosaic MUT mice with a nuclear envelope localized green fluorescent protein (GFP) Cre reporter (nTnG). Proximal tubule is marked by Villin. Inset images show the positive nuclei identified by U-Net image segmentation in solid green and GFP negative nuclei as gray outlines. Scale bars: whole kidney, 1 mm; inset image, 100 μm. *B*: Quantification of relative nuclear GFP in Villin+ proximal tubules in each segment for 12-wk-old mice. Each point represents the average fraction of all Villin+ nuclei in each segment for a single animal (*: $P = 0.0001$, 2-way ANOVA with Holm-Sidak's multiple comparison test with log transformation correction for heteroscedasticity, N = 10-12 mice). *C*: Quantification of relative nuclear GFP in Villin+ proximal tubules in each segment for 4-wk-old mice. (*: $P < 0.0001$, 2-way ANOVA with Holm-Sidak's multiple comparison test with log transformation correction for heteroscedasticity, N = 9-14 mice). *D*: Quantification of GFP+ cells in Villin+ proximal tubules for 4-wk-old mice membrane GFP reporter mice. (#: $P < 0.0001$, MUT S1/S2 vs S3, $P = 0.21$ for WT vs MUT in S1/S2, $P = 0.21$ WT vs MUT in S3, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 9 mice).

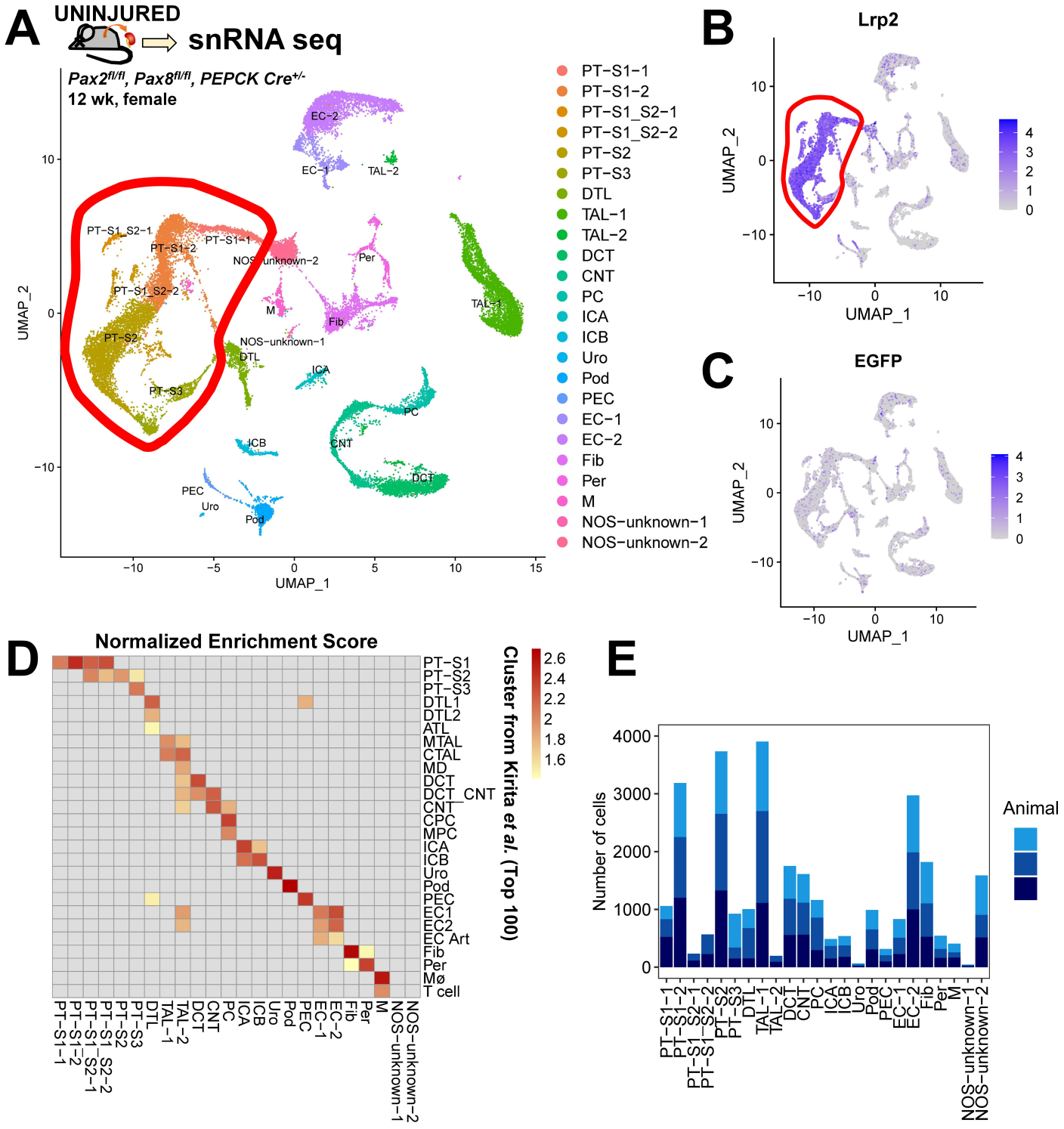

A
WT
Villin
GFP
Ki67
GFP
Ki67
Villin
MUT
Villin
GFP
Ki67
GFP
Ki67
Villin


B
Villin+ Proximal Tubule Cells
All PT Cells
Ki67+ Fraction
0.04
0.03
0.02
0.01
0.00
WT
MUT
*
*
S1/S2
S3
S1/S2
MUT
WT
**
ns
GFP+
GFP-
S3
MUT
WT
***
ns
GFP+
GFP-

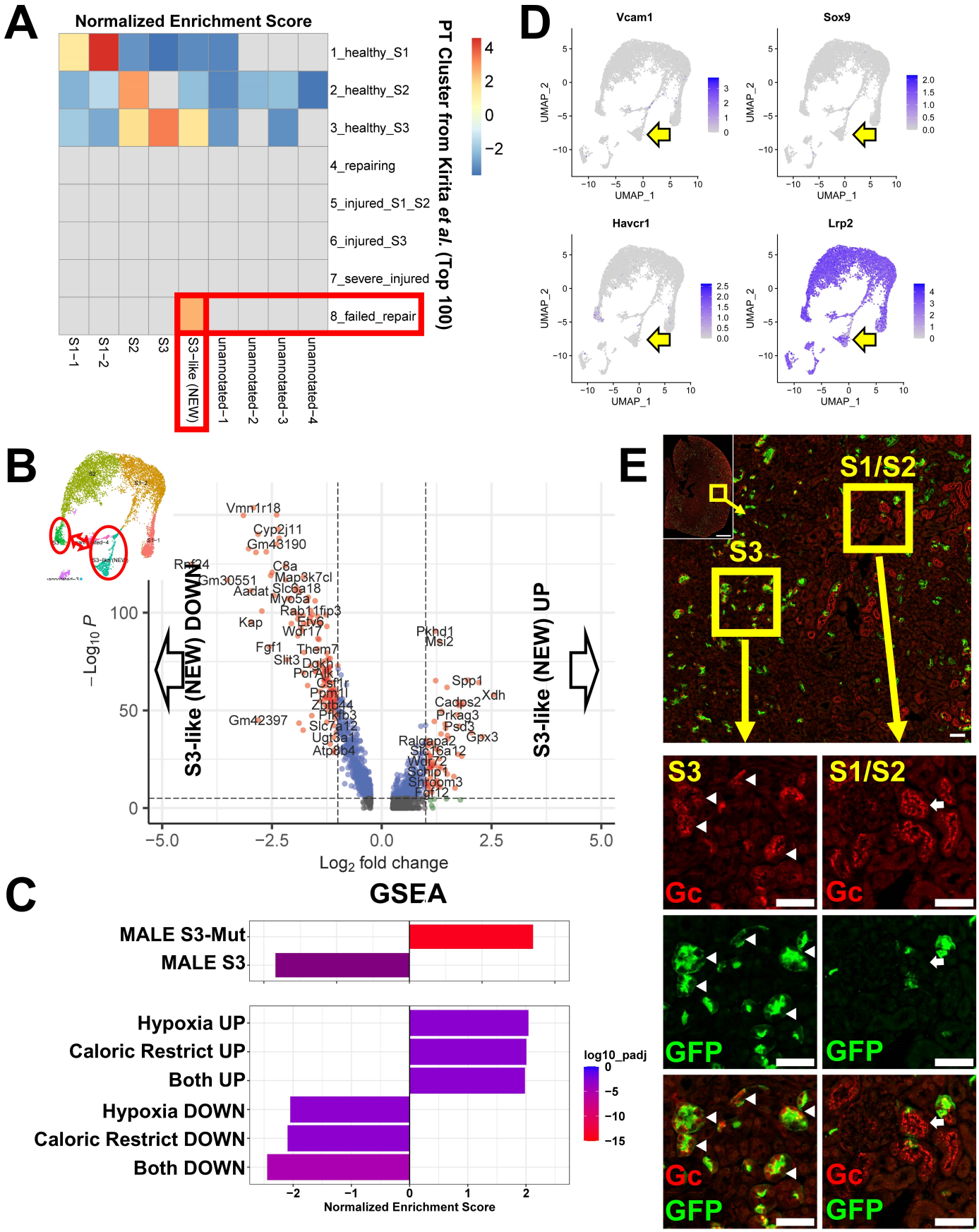
Supplemental Fig. S4. Mosaic mutant mice have higher baseline proliferation. *A*: Representative images of baseline proliferation marked by Ki67 staining in the S3 segment of the proximal tubules (PT) of mosaic wild type (WT) and mosaic mutant (MUT) mice. Only the S3 segment is shown because Ki67 staining was sparse, but higher in this segment in both strains. Scale bars: whole kidney, 1 mm; inset images, 50 μm. *B*: Quantification of the fraction of Villin+ proximal tubule cells that co-stain for Ki67 identified using U-Net image segmentation, overall or subdivided by proximal tubule segment. Each point represents the average fraction of all Villin+ nuclei in each segment for a single animal. (*: $P = 0.049$, *: $P = 0.015$, **: $P = 0.001$, ***: $P = 0.0007$, for All PT cells S1/S2, All PT Cells S3, S1/S2 GFP+, and S3 GFP+ respectively, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 5-10 mice).


Pax2
WT
S1/S2
S3
S1/S2
S3
Villin
GFP
Pax2
Merge
Pax2 (U-Net)
MUT
S1/S2
S3
S1/S2
S3
Villin
GFP
Pax2
Merge
Pax2 (U-Net)
Pax8
WT
S1/S2
S3
S1/S2
S3
Villin
GFP
Pax8
Merge
Pax8 (U-Net)
MUT
S1/S2
S3
S1/S2
S3
Villin
GFP
Pax8
Merge
Pax8 (U-Net)

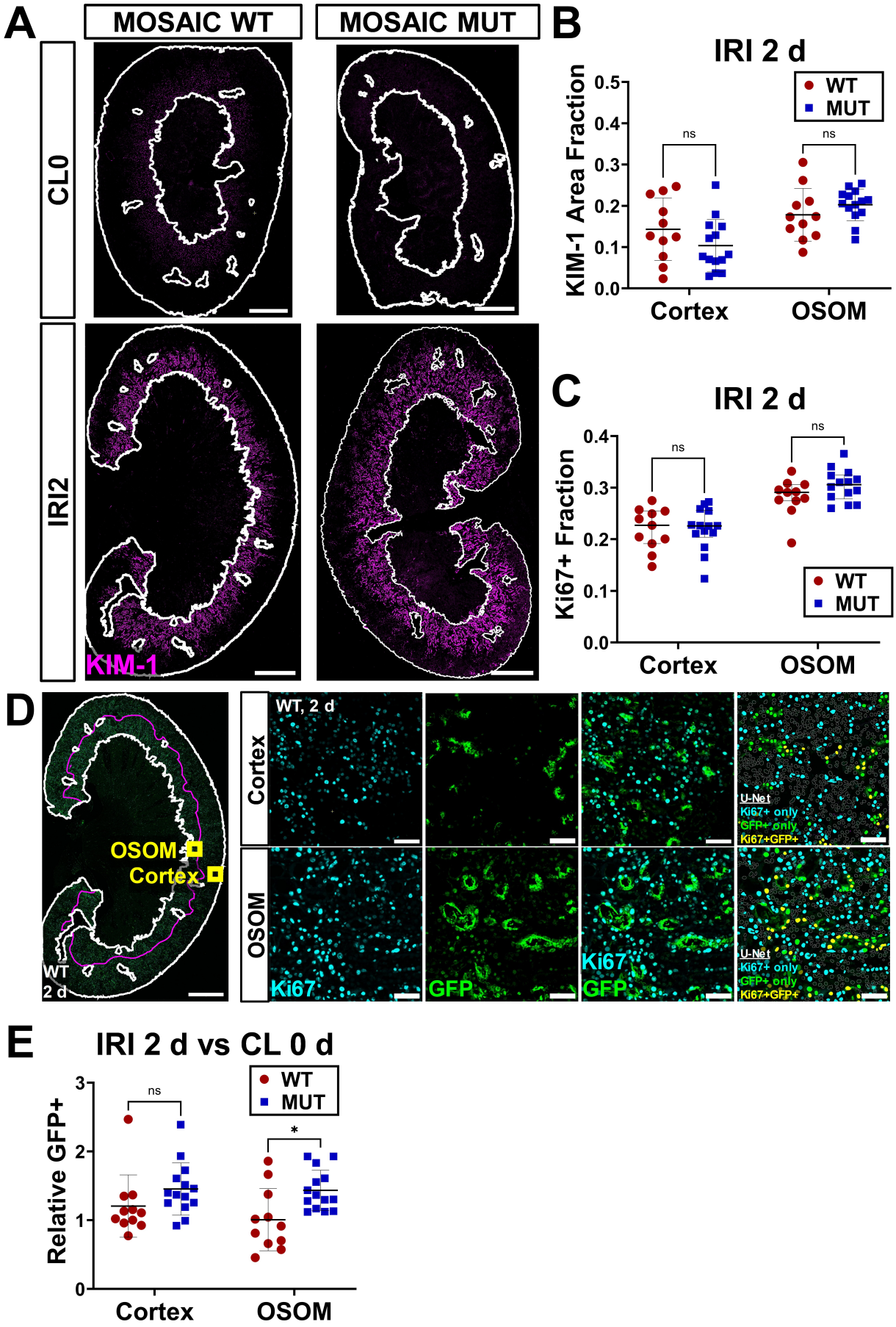
Supplemental Fig. S5. Green fluorescent protein (GFP) marks cells with depletion of Pax proteins in mosaic mutant (MUT) mice. Additional representative images of Pax2 (top) and Pax8 (bottom) staining in Villin+ proximal tubule cells by GFP status. In the whole kidney images, the white line indicates the analyzed cortex and outer stripe of the outer medulla (OSOM) and the magenta line indicates the S3 border. The rightmost image of each set shows U-Net identified Pax-positive nuclei. Gray outlines show negative nuclei. Arrowheads indicate Pax-depleted GFP+ proximal tubule cells. Scale bars: whole kidney, 1 mm; inset image, 50 μm.



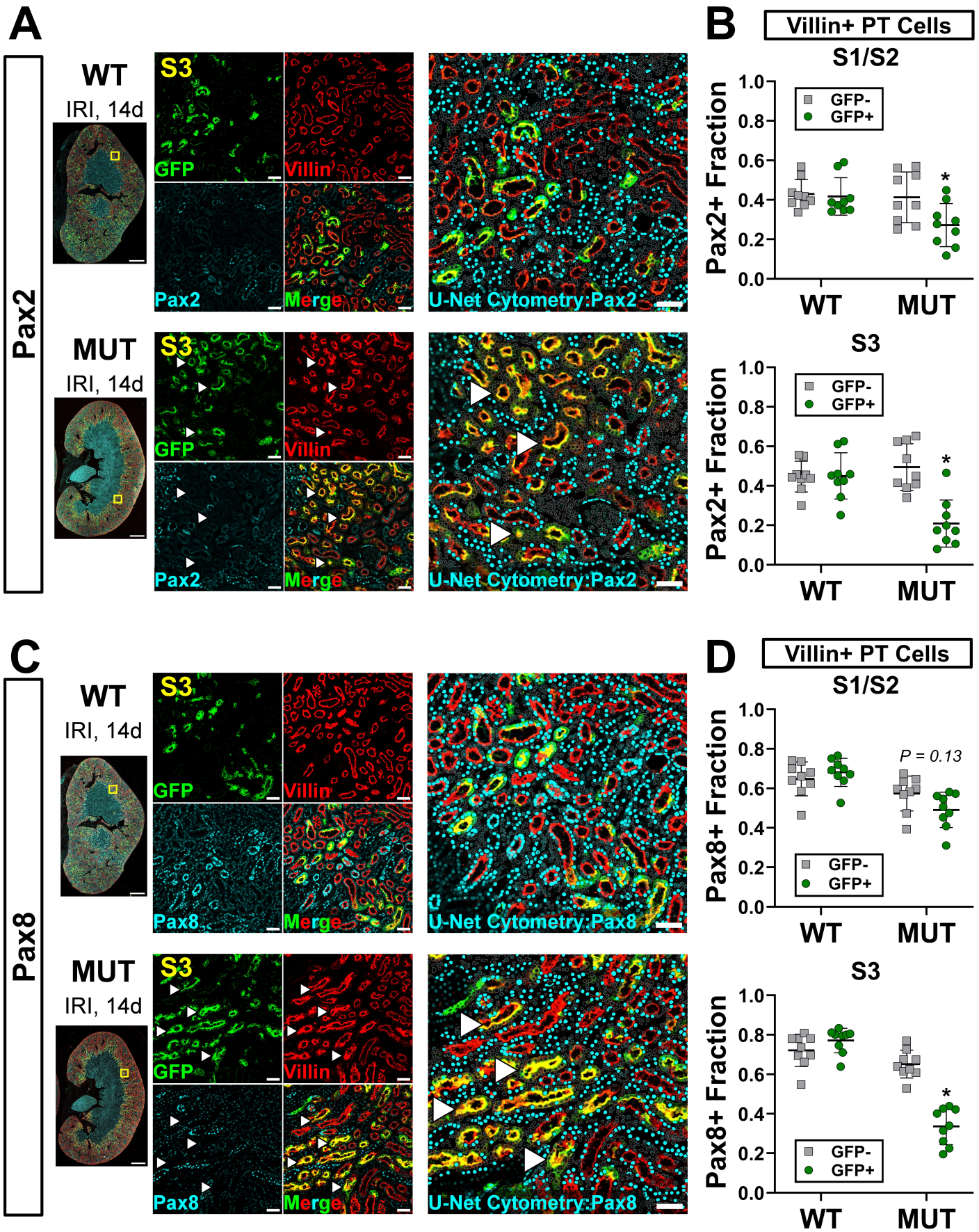
Supplemental Fig. S6. Single nucleus RNA sequencing (snRNA-seq) of uninjured mosaic mutant mice. *A*: Uniform manifold approximation and projection (UMAP) and clustering for all nuclei. Red outline indicates proximal tubule clusters extracted for further analysis in Fig. 4. *B*: Feature plot for *Lrp2* expression highlighting the proximal tubule clusters. *C*: Feature plot for EGFP expression, which included the 5' untranslated region in read mapping showing lack of clear mapping. *D*: Heat map of Geneset Enrichment Analysis (GSEA) of marker genes identified in our study against marker genes identified by Kirita, *et al*. (6) used to assign cluster identities. The normalized enrichment score, which is a metric of the degree of geneset enrichment, is shown. Gray squares indicate comparisons with adjusted $P > 0.05$. *E*: The contribution of nuclei to each cluster from the 3 animals included in the study showing uniform coverage from each sample.


A
Normalized Enrichment Score
PT Cluster from Kirita et al. (Top 100)
1_healthy_S1
2_healthy_S2
3_healthy_S3
4_repairing
5_injured_S1_S2
6_injured_S3
7_severe_injured
8_failed_repair
S1−1
S1−2
S2
S3
S3−like (NEW)
unannotated−1
unannotated−2
unannotated−3
unannotated−4
D
Vcam1
Sox9
Havcr1
Lrp2
UMAP_1
UMAP_2
B
S3-like (NEW) DOWN
S3-like (NEW) UP
−Log10 P
Log2 fold change
Vmn1r18
Cyp2j11
Gm43190
Rnf24
C8a
Gm30551
Map3k7cl
Aadat
Slc6a18
Myo5a
Rab11fip3
Kap
Etv6
Wdr17
Fgf1
Them7
Slit3
Dgkh
PorAlk
Csf1r
Ppm1l
Zbtb44
Prkdb3
Gm42397
Slc7a12
Ugt3a1
Atp8b4
Pkhd1
Msi2
Spp1
Xdh
Cadps2
Prkag3
Psd3
Gpx3
Raldapa2
Slc16a12
Wdr72
Schip1
Shroom3
Fgf12
C
GSEA
MALE S3-Mut
MALE S3
Hypoxia UP
Caloric Restrict UP
Both UP
Hypoxia DOWN
Caloric Restrict DOWN
Both DOWN
log10_padj
Normalized Enrichment Score
E
S1/S2
S3
Gc
GFP

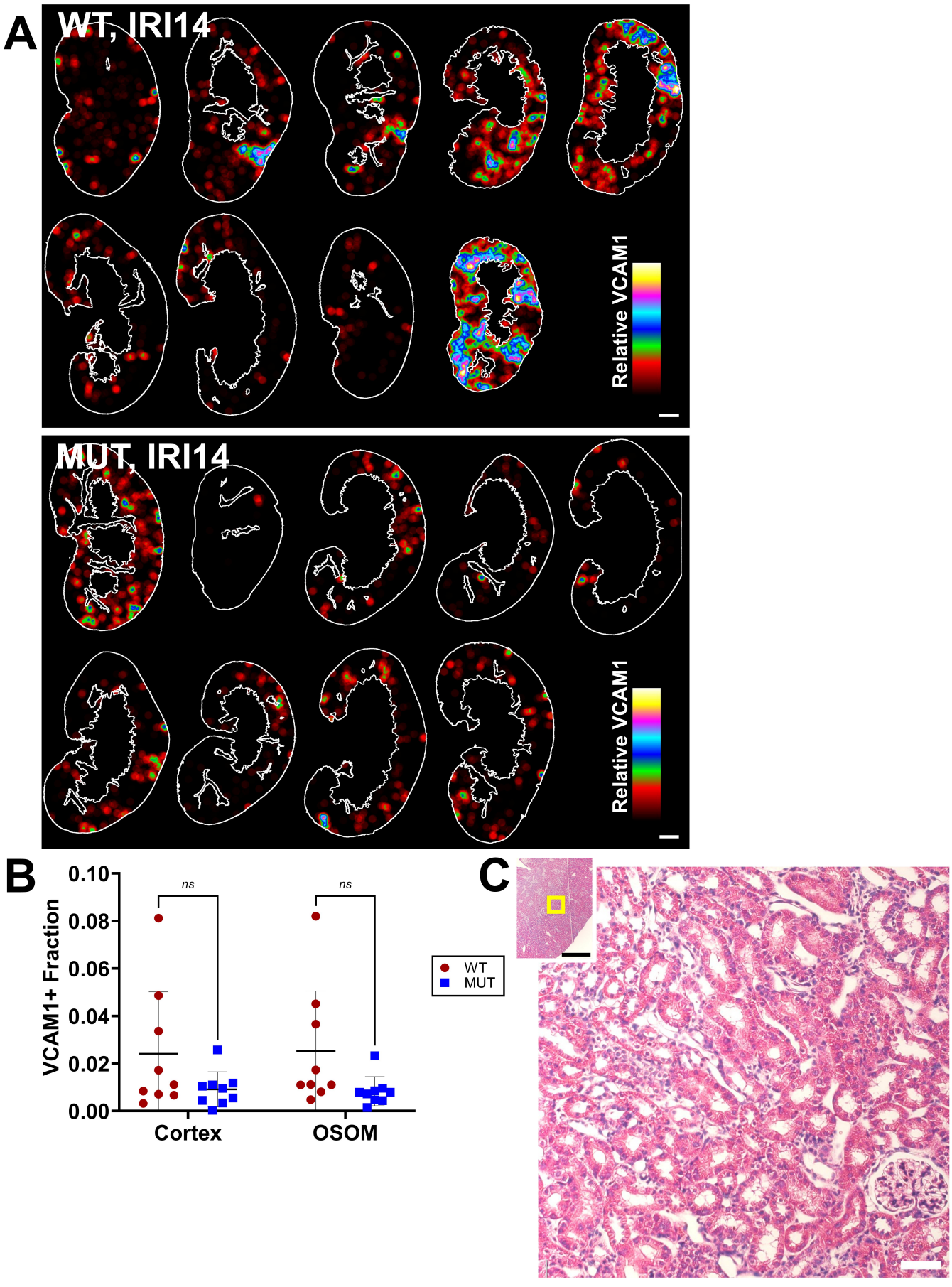
Supplemental Fig. S7. Mutant proximal tubule cells develop a gene expression signature that overlaps with chronic injury and preconditioning. *A*: Geneset Enrichment Analysis (GSEA) was performed against the top 100 proximal tubule cluster-specific marker genes identified in a benchmark dataset derived by Kirita, *et al.* (6) The normalized enrichment score was used as a metric of enrichment. Gray boxes denote comparisons with an adjusted $P > 0.05$. The new proximal tubule S3 cluster is strongly enriched with genes associated with chronic injury, termed “failed repair” by Kirita, *et al*. *B*: Volcano plot showing differentially expressed genes between normal and mutant S3 proximal tubule cells. *C*: Geneset Enrichment Analysis (GSEA) of differentially expressed genes compared with male Pax-mutant S3 proximal tubule cells (25) and in kidneys subjected to hypoxic or caloric restriction preconditioning (26). *D*: Feature plots for *Vcam1*, *Havcr1*, *Sox9*, and *Lrp2*. Arrows indicate the new S3 cluster. *E*: S3 vs S1/S2 staining for Gc protein from Fig 4 in mosaic mutant mice. In S3, there is a 1:1 relationship between cytoplasmic Gc protein and GFP in the S3 segment as well as mosaicism within the tubules (arrowheads). In the S1/S2 segments, Gc protein aggregates in discrete condensates near the brush border, which is consistent with resorption droplets (arrow). Also, there is no correlation with GFP+ cells nor any indication of mosaicism within the tubules. Scale bars: whole kidney, 1 mm; inset images, 50 μm.


A
MOSAIC WT
MOSAIC MUT
CL0
IRI2
KIM-1
B
IRI 2 d
KIM-1 Area Fraction
WT
MUT
ns
ns
Cortex
OSOM
C
IRI 2 d
Ki67+ Fraction
ns
ns
WT
MUT
Cortex
OSOM
D
OSOM
Cortex
WT
2 d
WT, 2 d
Cortex
OSOM
Ki67
GFP
Ki67
GFP
U-Net
Ki67+ only
GFP+ only
Ki67+GFP+
E
IRI 2 d vs CL 0 d
Relative GFP+
WT
MUT
ns
*
Cortex
OSOM

Supplemental Fig. S8. Mosaic mutant (MUT) and mosaic wild type (WT) mice develop similar acute injury. *A*: Representative whole section images of kidney injury molecule 1 (KIM-1) staining of uninjured contralateral kidneys excised at the time of surgery (CL0) and 2 d after ischemia reperfusion injury (IRI2). Scale bar: 1 mm. *B*: Quantification of the KIM-1 staining area using U-Net segmentation subdivided by cortex and outer strip of outer medulla (OSOM, statistical analysis: $P > 0.30$, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 11-14 mice). *C*: Quantification of the Ki67+ cell fraction U-Net segmentation subdivided by cortex and OSOM (statistical analysis: $P > 0.27$, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 11-14 mice). *D*: Representative images of U-Net tissue cytometry for Ki67 2 d after IRI for mosaic WT mice corresponding to Fig. 5E. Scale bars: whole kidney, 1 mm; inset images, 50 µm. *E*: Summary of U-Net based tissue cytometry showing the fraction of GFP+ cells at 2 days after ischemia reperfusion injury (IRI2) relative to the fraction in each region in the same animal in the contralateral kidney excised at the time of surgery (CL0, *: $P = 0.047$, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 11-14 mice).


A
Pax2
WT
IRI, 14d
MUT
IRI, 14d
S3
GFP
Villin
Pax2
Merge
U-Net Cytometry:Pax2
B
Villin+ PT Cells
S1/S2
S3
Pax2+ Fraction
GFP-
GFP+
WT
MUT
C
Pax8
Pax8
D
P = 0.13

Supplemental Fig. S9. The post-injury expanded population of green fluorescent protein positive (GFP+) proximal tubule cells in mosaic mutant (MUT) mice are depleted of Pax proteins. *A*: Representative images of outer stripe of the outer medulla (OSOM) 14 d after ischemia-reperfusion injury (IRI14) showing expression of Villin, GFP, and Pax2. The larger inset image shows the output of U-Net based cell tissue cytometry. All detected Pax2+ nuclei are shown for clarity, irrespective of Villin or GFP status. However, analysis was performed only for those nuclei associated with Villin+ proximal tubules and subdivided by GFP status. Grey outlines represent nuclei that were negative for Pax2. Scale bars: whole kidney, 1 mm; inset images, 50 μm.*B*: Quantification of Pax2 expression in Villin+ proximal tubule cells subdivided by GFP status. Each point represents U-Net-assisted quantification for the entire indicated region for one animal (*: $P = 0.027$, $P < 0.0001$ for S1/S2 and S3 respectively, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 9). *C*: Representative images of outer stripe of the outer medulla (OSOM) 14 d after ischemia reperfusion injury (IRI) showing expression of Villin, GFP, and Pax8 in mosaic wild type (WT) and mosaic mutant (MUT) mice as in *A* above. Scale bars: whole kidney, 1 mm; inset images, 50 μm. *D*: Quantification of Pax8 expression in Villin+ proximal tubule cells subdivided by GFP status as in *B* above (*: $P < 0.0001$, 2-way ANOVA with Holm-Sidak's multiple comparison test, N = 9).


A
WT, IRI14
Relative VCAM1
MUT, IRI14
Relative VCAM1
B
VCAM1+ Fraction
0.10
0.08
0.06
0.04
0.02
0.00
ns
ns
Cortex
OSOM
WT
MUT
C

Supplemental Fig. S10. Mutant repopulated S3 segments do not express vascular cell adhesion molecule-1 (VCAM-1) after injury. *A*: Heatmaps of VCAM-1 expression in 14 d after ischemia reperfusion injury (IRI14). The cortex and outer stripe of the outer medulla (OSOM) were analyzed, outlined in white. Scale bar: 1 mm. *B*: Quantification of the fraction of VCAM-1 positive cells in cortex and OSOM (P = 0.11, 2-way ANOVA, log-transformed due to heteroscedasticity, with Holm-Sidak's multiple comparison test, N = 9). *C*: Hematoxylin and eosin-stained serial section highlighting the same region of outer stripe of outer the medulla shown in Fig. 6*C*. Scale bars: low magnification image, 1 mm; region of interest, 50 μm.