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Supplemental Information

A Subpopulation of Label-Retaining Cells of the Kidney Papilla Regenerates Injured Kidney Medullary Tubules

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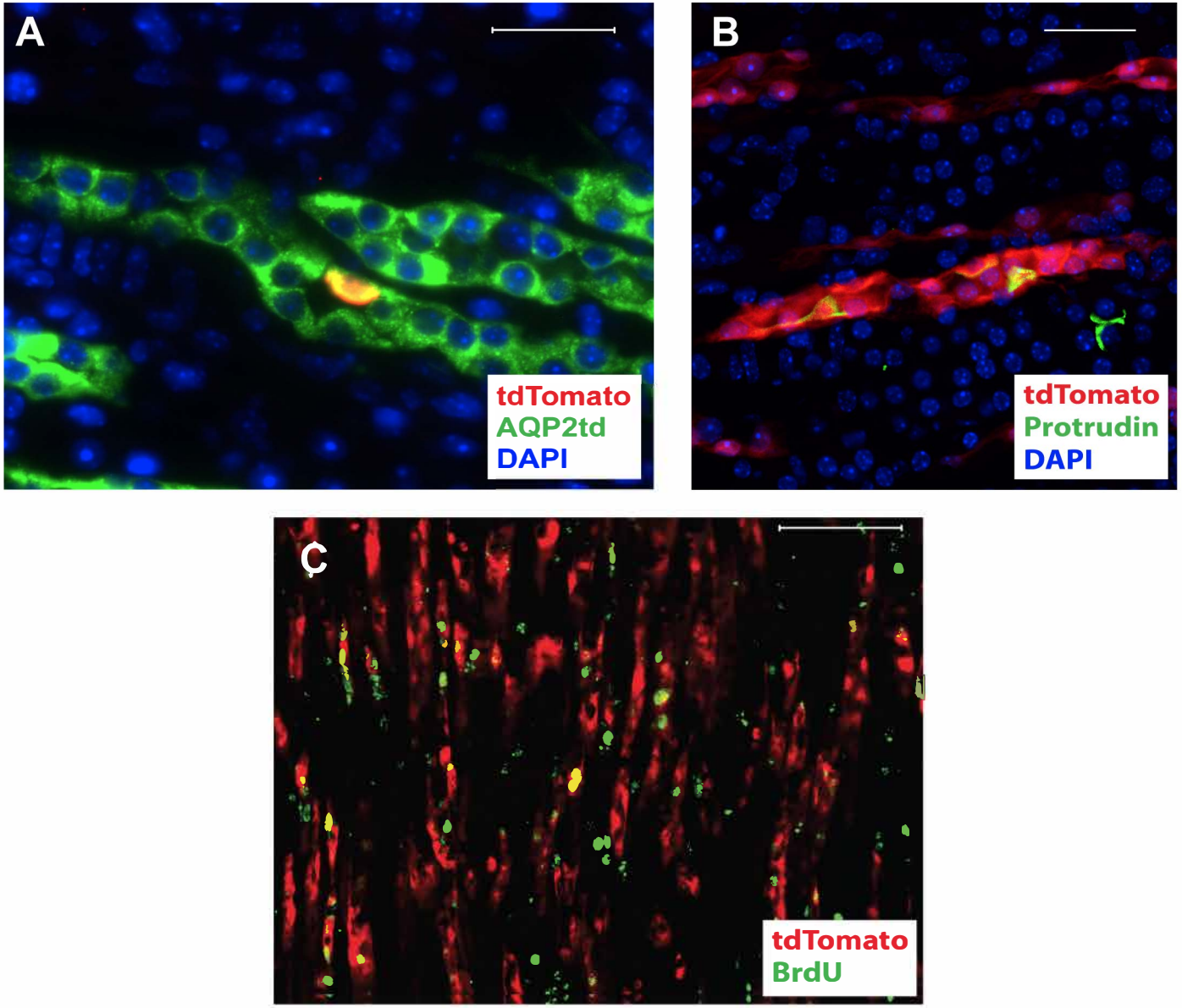
Figure Sn 1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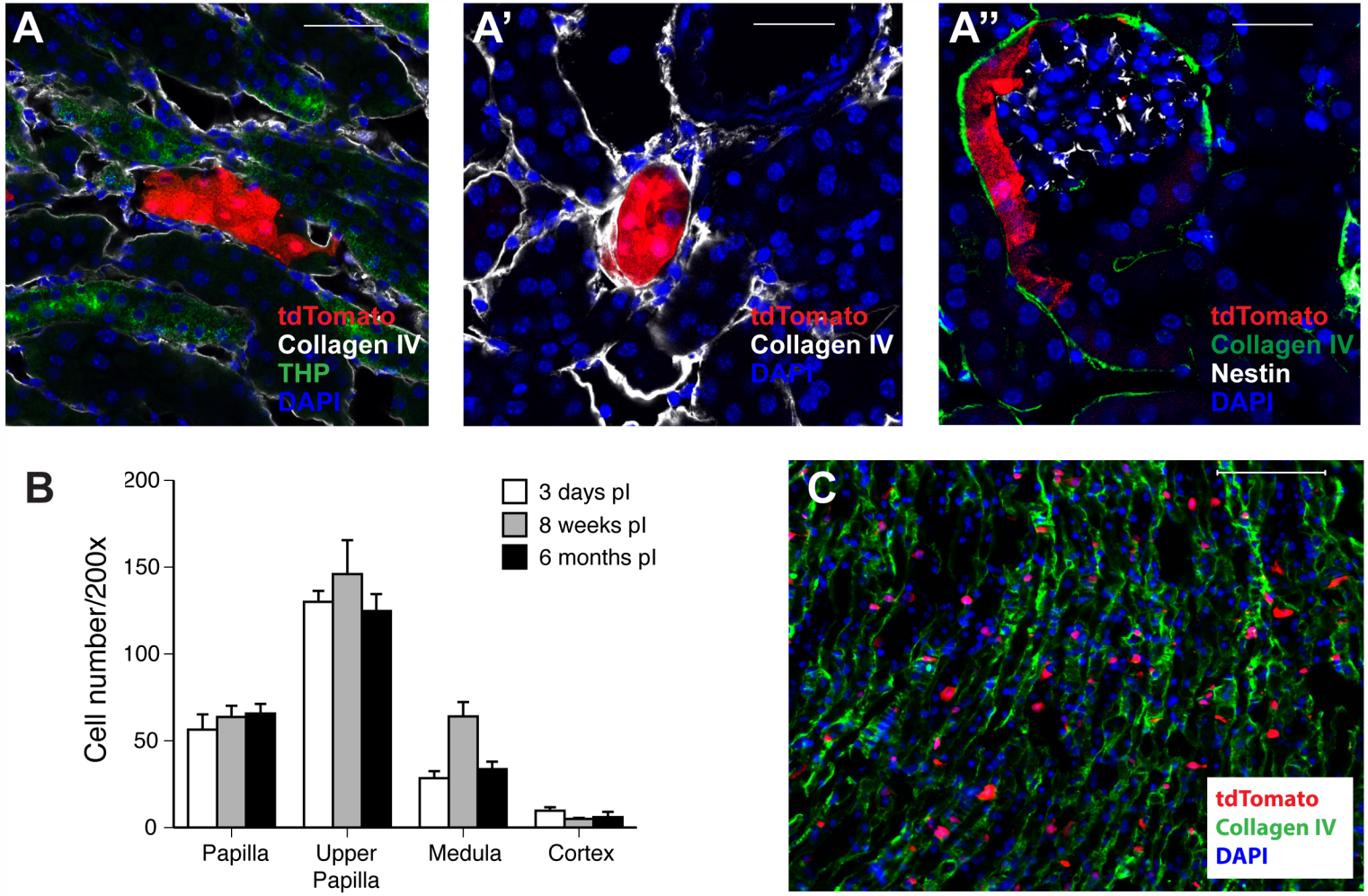
AQP2td
DAPI
B
tdTomato
Protrudin
DAPI
C
tdTomato
BrdU

Figure Sn 2


A
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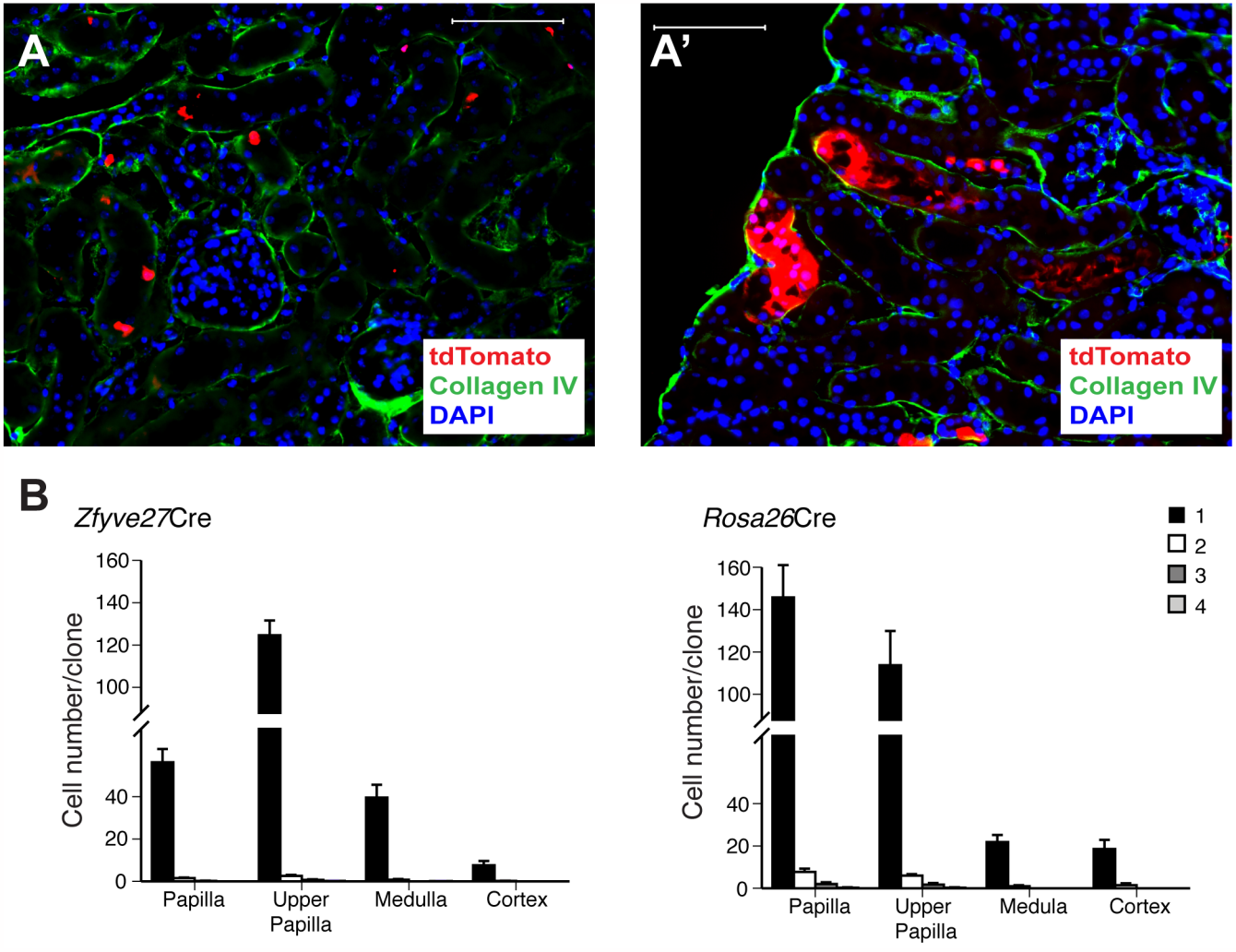
Collagen IV
THP
DAPI
A'
tdTomato
Collagen IV
DAPI
A''
tdTomato
Collagen IV
Nestin
DAPI
B
Cell number/200x
200
150
100
50
0
3 days pI
8 weeks pI
6 months pI
Papilla
Upper Papilla
Medula
Cortex
C
tdTomato
Collagen IV
DAPI

Figure Sn 3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tdTomato
Collagen IV
DAPI
A'
tdTomato
Collagen IV
DAPI
B
Zfyve27Cre
Cell number/clone
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140
120
100
40
20
0
Papilla
Upper Papilla
Medulla
Cortex
Rosa26Cre
1
2
3
4
Cell number/clone
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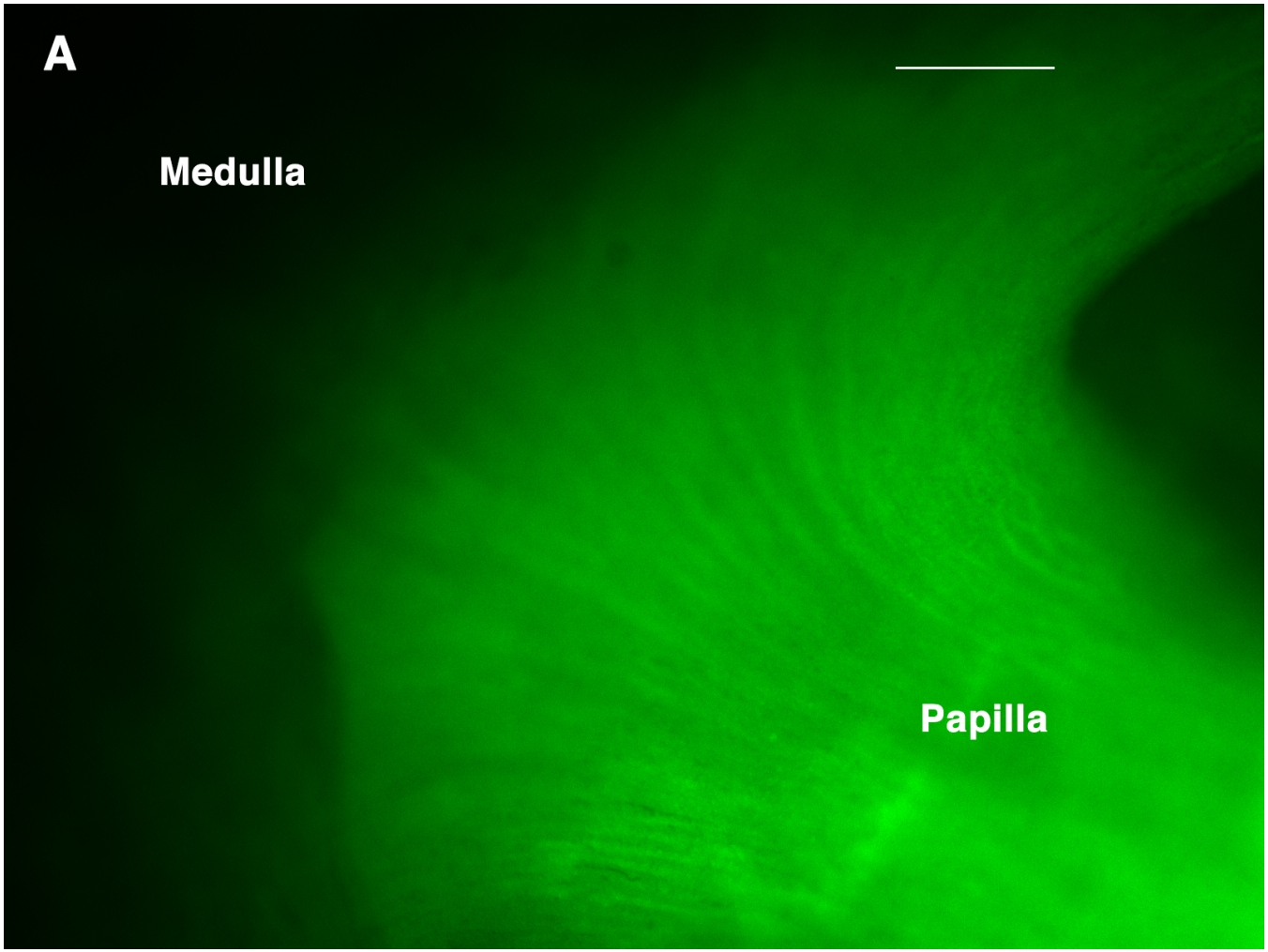
140
120
100
40
20
0
Papilla
Upper Papilla
Medula
Cortex

Figure Sn 4



SUPPLEMENTAL FIGURE LEGENDS

Figure S1. A. *Zfyve27*-Cre*ERT2*-marked cells. 25% of *Zfyve27*-Cre*ERT2*-marked cells in the papilla were in collecting ducts (n=5 mice); i.e., were positive for AQP2, as shown. 2 weeks pTM. Scale bar 25μm. **B. *Six2*-derived cells express protrudin**. 14% of *Six2*Cre-marked cells expressing tdTomato in the papilla expressed protruding (n=4 mice). Scale bar 25 μm**. C**. **Papillary *Six2*-derived cells are pLRCs.**. Kidney papilla of *Six2*Cre;*Rosa26*-tdTomato mouse given BrdU at 3 days of age and chased for 3 months. Note BrdU-retention in tdTomato marked cells (i.e., *Six2*-derived). n = 3 mice; scale bar 50 μm.

Figure S2. Tracing events by *Zfyve27*-Cre*ERT2*-marked cells in homeostasis. A, A', A''. Rare tracing events by *Zfyve27*-CreERT2-marked cells in mice > 5 months pTM. **A.** Tubular segment made of *Zfyve27-CreERT2*-marked cells in the medulla; THP, Tamm Horsfall Protein (Scale bar 40 μm). **A'**. Transverse cut of a tubular segment made of *Zfyve27-CreERT2*-marked cells in the kidney medulla. Scale bar 40 μm. A''. Bowman's capsule with multiple contiguous *Zfyve27*-CreERT2-marked cells; nestin staining identifies podocytes. Scale bar 40 μm. **B**. ***Zfyve27-CreERT2*-marked cells after 5 days at 3 days, 8 weeks and 6 months pTM**. The number of cells in all regions did not significantly increase from those at 3 days post tamoxifen; n=3 to 8 mice; Mean±SE. **C.** Upper papilla. As illustrated, *Zfyve27*CreERT2-marked cells in the upper papilla (8 days pTM) were, except for very rare exceptions, at clonal density. Scale bar 50 μm.

Figure S3. *Rosa26*-Cre*ERT2*T-marked cells. A. In *Rosa26-CreERT2;R*-tdTomato mice 8 days pTM (5 mg / Kg) marked cells were at clonal, or near clonal density. Scale bar 50 μm. **A'.** In these mice however, tamoxifen (even at 10x lower doses) occasionally marked groups of cells (8 days pTM). Scale bar 50 μm. **B. Clonal analysis of tdTomato$^+$ cells in the non-injured kidney of *Zfyve27-CreERT2;R*-tdTomato (n=5) and *Rosa26-CreERT2;R*-tdTomato (n=6) 16 days pTM**. As shown, in all

regions of the kidney, most of the *Zfyve27*CreERT2-marked cells were single clones (97 %; left). In the kidneys with *Rosa26-CreERT2*-marked cells, 92 % of the cells were single clones (right). Mean±SE. The number of clones in different kidney regions is shown in the Y-axis and the 1 to 4 keys indicate number of cells per clone.

Figure S4. Kidney organ culture and migration of *Zfyve27*CreERT2-marked cells **A.** Kidney medulla and papilla of a *Zfyve27-CreERT2;R*-tdTomato mouse administered FITC-Dextran 8 days pTM. FICT-Dextran 10 min before kidney isolation labeled the kidney papilla but not medulla allowing spatial information about the cell movement direction. Scale bar 250 μm. **B.** Migration of *Zfyve27*CreERT2-marked cells in the upper papilla. The upper papilla was analyzed with 27 cuts of 3 μm thickness captured every 5 min during 8.2 h. The movie shows the maximum intensity projection of the Z series.

Table S1. qPCR fold change of candidate genes normalized to GAPDH

Gene Symbol	Fold overexpression	Standard deviation (+/-)
Bst1	17.4	2.1
Clk1	15.6	2.0
Cxcr4	9.1	1.6
Dach1	9.3	2.2
Dlg1	12.8	2.1
Dlg5	16.4	2.3
Dlg7	7.0	1.3
Erap1	8.6	1.4
Esr1	18.9	4.1
Fancd2	9.9	1.6
Flot1	8.2	1.5
Foxg1	11.3	1.4
G3bp1	22.7	3.9
Hoxa9	14.5	2.7
Hoxb5	12.5	1.9
Ly6e	17.7	2.7
Malat1	16.8	2.6
Ogt	14.9	1.8
Pax2	7.2	1.1
Podn	19.3	3.0
Ryk	8.3	1.9
Sssca1	12.9	2.2
Sfrs1	15.4	2.4
Syne2	5.8	0.8
Tm2d3	15.3	2.2
Tsg101	15.1	1.8
Tspan3	13.4	1.8
Tspan6	10.6	1.9
Zfyve27	13.3	1.6
Zmpste24	12.4	1.2

Table S2. Detection of proteins encoded by candidate genes by immunofluorescence microscopy in the Kidney.

Gene	*Result*
NPD	Collecting system
SGCB	Non-specific
TSG101	Negative
ERAP1	Non-specific, glomerulus, tubules
FLOT1	Few in papilla and medulla
CLK1	Non-specific
SFRS1	Non-specific
HOXA9	No papillary staining
HOXB9	Negative
SF2	Negative
ESRA	Papilla, medulla, uro-epithelium
TSG101	Negative
OGT	Non-specific
DACH1	Nuclear, mainly in papilla
ZMPSTE24	Negative
LY6A/E	Non-specific
GP130	Uro-epithelial, tubules and glomerulus
LGR5	Negative
ZYVE27	Papilla only

Table S3. Fraction (%) of *Zyve27*-CreERT2-marked; i.e., tdTomato^{-} and tdTomato^{+} cells in kidney papilla that expressed cell surface by flow cytometry analysis.

	tdTomato ^{negative}	tdTomato ^{positive}
CD24	94	100
CD29 (β1 integrin)	54	86
CD49f (α6 integrin)	36	57
CD133	30	53
SCA-1	73	78
CXCR4	22	54
CXCR7	56	70
CXCR4 + CXCR77	13	43

All data points from 3-4 independent experiments and each experiment contained cells from 2 mice. Isotype-specific antibodies were used as controls.

Table S4. Clonal analysis of *Zfyve27*CreERT2-marked cells in the kidneys of *Zfyve27-CreERT2;R*-tdTomato mice after one dose of tamoxifen (40 mg / Kg).

Kidney region	Total Cell number	Single clones	Double clones	% Single clones
Cortex	60	60	0	100
Medulla	166	164	1	99
Upper papilla	816	806	4	99
Papilla	155	155	0	100

n=6 mice; data obtained from 3-21 days pTM.

Table S5. Concentrations of Tamoxifen (TM) and 4-Hydroxytamoxifen (4-HO-TM) in mouse kidney papilla and in the cortex plus medulla.

	TM			4HO-TM	
	Papilla	Cortex+Medulla		Papilla	Cortex+Medulla
Exp #1	406.2	901.9		64.6	140.0
Exp #2	280.0	1142.3		37.9	150.7
Average	343.1	1022.1		51.3	145.4

The *Rosa26*-CreERT*2;R*-tdTomato bi-transgenic mice had many marked cells in the kidney papilla after 5 mg/Kg TM (see Figure 3A). We tested whether papillary accumulation of TM and/or its most active metabolite 4-Hydroxytamoxifen could account for this observation by measuring their concentration by liquid chromatography–mass spectrometry**.** However, as shown in the table, the concentration of both compounds was markedly lower in the papilla than in the rest of the kidney and the reason why these mice had such abundance of tdTomato^{+} cells in the papilla is unclear. For each experiment, 5 mice were given 40 mg / Kg TM for 5 consecutive days and euthanized 3 days later.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genome expression analysis of papillary LRCs

Gene expression analysis of the pLRCs from the H2B-GFP mouse (GFP^+ vs GFP-negative papillary cells) found 6,400 significantly up-regulated or down-regulated genes (fold-change greater than 2 or lower than 2 and $p< 0.01$). Output data including gene lists with complete raw data sets have been deposited in (GEO ID: GSE71693). We narrowed the candidate gene list to include those with fold-change greater than or lower than 5. Of these potential candidates, manual extraction revealed that 216 of 2713 transcripts (8%) were consistently over-expressed in the papillary LRCs versus the GFP-negative cells. We confirmed the over-expression of 30 of the most highly over-expressed genes in the LRCs versus the GFP-negative cells by first using the MessageBOOSTER cDNA Synthesis Kit (Epicentre, Madison, Wisconsin) to amplify mRNA and then by performing qPCR. Experiments were run in triplicate and fold-expression changes are shown in Table S1.

Mice

Mice were maintained at the Columbia University Medical Center Institute of Comparative Medicine, in accordance with its guidelines and all experiments were performed with approval of the IRB of Columbia University. H2B-GFP mice were as described (Oliver et al., 2009). Briefly, to obtain a line where administration of doxycycline caused expression of the fusion protein histone2B-GFP we used the previously published TetO-H2BGFP line (Tumbar et al., 2004) but instead of crossing it with a line with a keratin5-tTA driver, we crossed it with a *Rosa26*-rtTA line (Yu et al., 2005). *Rosa26*-rtTA;teto-H2BGFP bi-transgenic offspring did not express GFP but doxycycline administration through drinking water resulted in expression of histone2B-GFP (Oliver et al., 2009). The fusion protein histone2B-GFP is stable for at least six months in post-mitotic cells (e.g., photoreceptors) and in cycling cells it dilutes into their progeny (Brennand et al, 2007).

All other mice were obtained from Jackson Laboratories, specifically: the reporter mouse line B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (Number: 007914), the universal Cre mouse line B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J (Number: 008463), the *Six2* Cre line STOCK Tg(Six2-EGFP/cre)1Amc/J (Number 009606) and C57BL/6J (Number: 000664). For all these mouse lines, genotyping was performed as recommended by Jackson Laboratories.

Tamoxifen administration

Tamoxifen (Sigma) was prepared as described (Metzeger & Chambon 2001) and administered by intra-peritoneal injection at 40 mg/Kg once, unless specified. To determine the duration of CreERT2 action in the kidney after tamoxifen, we administered tamoxifen to 15 *Zfyve27*-CreERT2;*R*-tdTomato mice and sacrificed 3 mice each at days 1, 2, 3, 4 and 5 pTM. Quantification of all tdTomato^{+} cells showed that there were abundant cells at 24 h pTM, that their numbers increased slightly at 2 and 3 days but remained unchanged between days 3, 4 and 5 days pTM.

EdU administration

For normal mice, was dissolved in PBS at 8 mg/ml. This solution was introduced into an Alzet osmotic mini-pump (Number 2002) and implanted subcutaneously to *Zfyve27*-CreERT2;R-tdTomato adult mice under general anesthesia. During pump implantation, the mice were given 100 µg of EdU by subcutaneous injection. The mini-pumps delivered 96 µg of EdU daily for two weeks, at which time the mice were sacrificed and their kidneys harvested. Mice subjected to renal artery occlusion in order to injury the kidney were given EdU (dissolved in PBS at 2 g/ml) as a subcutaneous injection of 50 µg/gr at 8 AM and 6 PM the day before and at 8 AM the day of sacrificed, which took place 6 h after the last EdU injection.

Ischemic kidney injury (KI)

KI was induced as previously described by clamping the left renal artery (Oliver et al., 2009) for the specified time periods. Most KI experiments were done at least one week pTM. In a few instances, we performed KI 3 or 4 days pTM since after 3 days we found no evidence of CreERT2 activation (see above). The time interval between TM administration and KI is specified in the legends of all described experiments and it is expressed as time pTM; i.e., post-tamoxifen.

Immuno-detection and confocal microscopy

Kidneys were isolated and processed as described (Oliver et al., 2009). Most kidney sections were of 5 μm but 10-15 μm sections were obtained for confocal analysis. BrdU (Life Technologies) detection was done as previously described (Oliver et al., 2004), except where indicated. EdU (Life Technologies) was detected following the manufacturer protocol**.** Fluorescence signals were detected with a fluorescence microscope and an RT Slider SPOT digital camera (Diagnostic Instruments) and with an Axiovert 100 laser-scanning confocal microscope (model LSM 410; Carl Zeiss).

BrdU-detection in kidneys with tdTomato$^+$ cells. Immuno-detection of BrdU in kidney sections requires their incubation with HCl for ~ 30 min to allow effective antibody penetration. This resulted in loss of the tdTomato signal and a series of experiments was carried out to develop a procedure that attained effective anti-BrdU antibody penetration but retained the tdTomato signal. We found that by first treating the kidney sections with Target Retrieval Solution (Dako), followed by only 10 min of HCl and standard neutralization with NaOH, anti BrdU antibody penetration was efficient and the tdTomato signal partially preserved (see Figure 1F).

Quantification of Zfyve27CreERT-marked cells. For each mouse, of 12 consecutive 5 μm sections containing a complete sagittal cut (and including the papilla) of one of the two kidneys, section numbers 1, 6 and 12 were selected for analysis after staining with DAPI and a collagen IV antibody. In the fluorescence microscope and guided by the image of the collagen IV staining (in FITC), from the cortex, medulla and upper papilla, three random fields were selected at 200x unless specified. For the

smaller main body of the papilla, only two fields were selected. After selection, a photograph with the Rhodamine filter was taken and the number of tdTomato$^+$ quantified.

Quantification of EdU positive cells. In mice with Zfyve27CreERT-marked cells and that received EdU, kidney sections were stained with DAPI and EdU visualized as instructed by the manufacturer (Life Technologies). Three non-contiguous sections were analyzed as described in Quantification of Zfyve27CreERT-marked cells above and the number of DAPI$^+$ and tdTomato negative cells and of the tdTomato$^+$ cells quantified. In these two populations the number of EdU positive nuclei was next obtained and expressed as percentage of the total cells.

Clonal analysis of Zfyve27CreERT-marked cells. For each mouse, all individual tdTomato$^+$ cells present in the cortex, medulla, upper papilla and papilla of a 5 μm section were identified by co-staining their nuclei with DAPI. Single clones were defined as a tdTomato$^+$ cell containing a single nucleus and not contiguous to any other tdTomato$^+$ cell. Double cell clones when two contiguous tdTomato$^+$ cells containing two nuclei stained with DAPI.

Quantification of new tubules made of tdTomato$^+$ cells. Both the un-injured (control) and injured kidneys of *Zfyve27*CreERT2;R-tdTomato and of ROSACreERT2;R-tdTomato mice were sagittal sectioned and the side containing the papilla was mounted for tissue sections. Twenty 5 μm sections containing a complete sagittal cut of the kidney were obtained and sections numbered 1, 10 and 20 were stained for collagen IV and examined under the fluorescent microscope. A tubule made up of tdTomato$^+$ cells was define as a group of these cells that made a circular tubular structure or > 10 tdTomato$^+$ longitudinally contiguous cells. For each kidney, all tubules present in a section were counted and averaged. Measurements of tubular length were done with ImageJ.

qPCR.

Was performed as described (Oliver et al., 2009).

Flow cytometry

Adult mice with Zfyve27CreERT-marked cells were sacrificed and the papillae of their kidneys isolated. After mincing and incubation with collagenase I (Worthington), papillary cells were isolated as described (Oliver 2004). Flow cytometry was carried as described (Oliver et al., 2009) except that given the intensity of the tdTomato signal, all antibodies (and appropriate isotype-specific controls) used were coupled to either APC or Alexa 647 and when an antibody couple to those fluors was not available, antibodies coupled to Pacific Blue were used.

Kidney organ culture and 2-photon microscopy

Because *Zfyve27* is also expressed in the bone marrow (see GeneAtlas U133A at http://biogps.org/#goto=genereport&id=118813) and given the kidney's extensive vascular network, we first determined how many of the *Zfyve27CreERT2*-marked cells in the kidney papilla were circulating cells originating in the bone marrow. To do this, we isolated papillary cell suspensions from *Zfyve27CreERT2*;*R*-tdTomato mice and probed them with a CD45-Alexa coupled antibody by flow cytometry. In three independent experiments, only 3.1 ± 0.2% (mean ± SE; n=6 mice) of all tdTomato$^+$ cells were also positive for CD45. Hence, a small fraction of the papillary tdTomato cells is of bone marrow origin.

Under general anesthesia, Zfyve27CreERT2;R-tdTomato mice previously treated with TM were given 5 mg of FITC-dextran (Life Technologies, D-1821) intravenously. After ~ 10 min, mice were sacrificed and one of their kidneys isolated. The FITC-dextran accumulated in the lower papilla (Lencer et al., 1990) and provided spatial orientation during microscopy (Figure S7). The kidney was sectioned by its sagittal plane so that the papilla was attached to only one kidney half. The sectioned surface of this half was then placed on a glass cover slide coated with Matrigel (BD Biosciences), covered with warmed DMEM containing 10% FCS and incubated for ~ 8 h at 37^{o}C in a 5% CO_2 atmosphere. After

incubation, the half kidney had attached to the Matrigel and was taken to the 2-photon microscope for analysis.

Kidneys were imaged by 2-photon excitation at 920 nm using a 25x/1.1 Apo-LWD water-immersion objective lens on a Nikon A1R MP multiphoton imaging system. Fluorescent proteins were detected with non-descanned detectors using standard green and red filters in the reflected light path. The culture dish was placed in a heated, humidified stagetop chamber with 5% CO_2 atmosphere. Cell movement was examined by obtaining serial 3 μm sections every 5 minutes for ~ 8 h while maintained the kidney in tissue culture conditions.

Antibodies

The antibodies used for immune-fluorescence were: AQP2, rabbit polyclonal (Sigma, A7310) and goat polyclonal (Abcam, ab105171); AQP1 rabbit polyclonal (Abcam; ab15080); BrdU, rat monoclonal (Abcam, ab6326); calbindin, rabbit polyclonal (Swant, CB38); CD11c rat monoclonal (clone M1/70, eBioscience, 14-0112-81); CD140b, rat monoclonal (clone APB5, eBioscience, 14-1402-81); CD146, rabbit monoclonal (Abcam, ab75769); CD68, rat monoclonal (Abcam, ab53444); CLC-K rabbit polyclonal (Santa Cruz, sc-292791), goat polyclonal (Santa Cruz, sc-21295) and rabbit polyclonal to CLC-K (Chemicon); collagen IV, rabbit polyclonal (Abcam, ab19808) and goat polyclonal (SouthernBiotech, 1340-01); megalin, goat polyclonal (Santa Cruz, sc-16478); p75NGFR, rabbit polyclonal (Abcam, ab38335); PAR3, rabbit polyclonal (Millipore; 07-330); perlecan, rat monoclonal A7L6 (Millipore, MAB1948P); PKC ζ, rabbit polyclonal (Santa Cruz; sc-216); protrudin, rabbit polyclonal (Santa Cruz, sc-102174) and rabbit polyclonal (Abbiotec, 252206); Sox9, rabbit polyclonal (Novus, NRP1-85551), Tamm Horsfall Protein sheep polyclonal (Chemicon, AB733). Nuclei were stained with DAPI (Invitrogen).

Antibodies used for flow cytometry were: APC rat anti-mouse CD45 (clone 30-F11, BioLegend) and V450 rat anti-mouse (BD, 560501); Alexa fluor 647 rat anti-mouse CD24 (clone M1/69, BioLegend, 101817); APC rat anti-mouse CD133 (clone 315-2C11, BioLegend, 141207); Alexa fluor 647 Armenian

hamster anti CD29 (clone HMβ1-1, BioLegend, 102213); Pacific Blue rat anti CD49f (clone GoH3, BioLegend, 313619); Alexa fluor 647 rat anti-mouse Ly-6A/E (Sca-1) (clone E13-161.7, BioLegend, 122517); Brilliant Viole 421 rat anti-mouse CD184 (clone 2B11/CXCR4, BD, 562738) and APC rat anti-mouse (clone 2B11, eBioscience, 17-9991-80); APC anti CXCR7 (clone 8F11-M16, BioLegend, 331113)

Reagents

Tamoxifen and 4-Hydroxytamoxifen were from Sigma; FITC-Dextran, 10,000 MW from Life Technologies (D-1821); EdU from Invitrogen (A10044); BrdU from Sigma (B9285) and Target Retrieval Solution from DAKO (S1699).

Liquid chromatography–mass spectrometry

Tissue samples were homogenized in water at a concentration of 1 mg/ml. Homogenates were extracted with 8 ml of hexane/methylene chloride: 3/2. Supernatants were evaporated under nitrogen and re-suspended in 50ul of 50 % methanol with 0.1 % formic acid. A Waters Acquity UPLC system with a 100 mm BEH C18 column was used for separation. The column was equilibrated with 75% water with 0.1% formic acid and 25% acetonitrile with 0.1% formic acid. A Waters XEVO TQS Tandem Mass Spectrometer was used for analyte detection. Our calibration curve was sensitive down to 25 pg /ml.

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