

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

Juan A. Oliver,^{1,*} Rosemary V. Sampogna,¹ Sumreen Jalal,¹ Qing-Yin Zhang,² Alexander Dahan,¹ Weiwei Wang,¹ Tian Huai Shen,¹ and Qais Al-Awqati^{1,3,*}

¹Department of Medicine

²Department of Pathology and Cell Biology

³Department of Physiology and Cellular Biophysics

Columbia University, New York, NY 10032, USA

*Correspondence: jao7@columbia.edu (J.A.O.), qa1@columbia.edu (Q.A.-A.)

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SUMMARY

To determine whether adult kidney papillary label-retaining cells (pLRCs) are specialized precursors, we analyzed their transcription profile. Among genes overexpressed in pLRCs, we selected candidate genes to perform qPCR and immunodetection of their encoded proteins. We found that *Zfyve27*, which encodes protrudin, identified a subpopulation of pLRCs. With *Zfyve27-CreERT2* transgenic and reporter mice we generated bitransgenic animals and performed cell-lineage analysis. Post tamoxifen, *Zfyve27-CreERT2* marked cells preferentially located in the upper part of the papilla. These cells were low cycling and did not generate progeny even after long-term observation, thus they did not appear to contribute to kidney homeostasis. However, after kidney injury, but only if severe, they activated a program of proliferation, migration, and morphogenesis generating multiple and long tubular segments. Remarkably these regenerated tubules were located preferentially in the kidney medulla, indicating that repair of injury in the kidney is regionally specified. These results suggest that different parts of the kidney have different progenitor cell pools.

INTRODUCTION

Epithelial organs such as the kidney appear to have a constant number of cells once they reach maturity. When cells die, adjacent terminally differentiated cells might divide within the plane of the epithelial sheet to replace them, but work in many organs indicates that often a more specialized pool of progenitor/stem cells exist to serve this function. To date, robust identification of progenitor/stem cells has required markers that are present in them but not in their surrounding cells and that, in addition, allow identification of their progeny. The function of many of these markers was largely unknown (at least initially); some had been cytoskeletal proteins; e.g., keratins (Rock et al., 2009), others were surface receptors such as LGR5 (Barker et al., 2007) or members of CD family, and many had nothing to do with “stemness.” Yet, with genetic cell-lineage tracing they opened the way for the next leap in analytical power. Introduction of a genetic label under the control of the marker’s promoter into the cells allowed identification of their in vivo location and, more significantly, permitted visualization of the contribution of single cells to multiple differentiated lineages in the same organ. Using this approach it was discovered that there appeared to be several stem cell pools in a given organ (Page et al., 2013; Donati and Watt, 2015); that there might be no obligatory hierarchy where a group of stem cells produced all differentiated subtypes during homeostasis (Sun et al., 2014), that there might be different stem cell pools that mediate homeostatic cell maintenance and organ regeneration (Tian

et al., 2011; Mascré et al., 2012; Vaughan et al., 2015), and that injury can change lineage-restricted progenitor cells so that they become true stem cells (Ito et al., 2007; van Es et al., 2012).

The adult mammalian kidney is an organ with very low cell cycling during homeostasis but remarkable proliferating capacity after injury. It is still unresolved whether the kidney contains bona fide stem cells. Humphreys et al. (2008) genetically marked cells using *Six2*, a transcription factor expressed in embryonic kidney epithelial stem cells, and fate-mapped their progeny during adult kidney regeneration; they found that new epithelial cells after injury derived from cells of the same embryonic lineage. Although this study was interpreted to indicate that stochastic proliferation of terminally differentiated cells generates the new cells needed for kidney repair and that thus the adult kidney does not contain stem cells, it could not exclude that resident stem cell pools in the adult kidney might themselves be derived from the *Six2* compartment. During kidney regeneration from injury, Berger et al. (2014) performed cell-lineage analysis of a postulated proximal tubular epithelial stem cell population that was genetically labeled by doxycycline administration. When labeling was done before kidney injury (KI) the labeled cells did not expand, suggesting that these scattered proximal tubular cells were not stem/precursor cells. Similarly, labeling proximal tubular cells before injury followed by injury showed that there was no dilution of the label, which was interpreted as favoring the absence of a progenitor pool (Kusaba et al., 2014). Cell-lineage tracing has also been



applied to investigate the origin of podocytes, a particular target of many kidney diseases. Several lines of evidence suggested that adult podocytes might derive from the parietal epithelial cells lining Bowman's capsule (Ronconi et al., 2009), and Appel et al. (2009) found that a transgenic mouse with podocalyxin (expected to identify podocytes) unexpectedly expressed the transgene in the parietal epithelial cells. Inducible gene tagging of these cells with doxycycline showed that they generated podocytes but only in mice of young age, a time when kidney size increases dramatically. More recently, Rinkevich et al. (2014) used an unbiased approach to mark single-cell clones in the adult kidney and found that they generated long tubular segments along the nephron, strongly suggesting the presence of specialized progenitor cells that were segment specific in the nephron.

To search for stem cells in the adult kidney, we originally used the observation that many organ-specific stem cells cycle at very low rates, and with S-phase markers identified a population of low-cycling cells in the adult kidney papilla (Oliver et al., 2004, 2009). Since the cells retain these markers for long periods, we termed them papillary label-retaining cells (pLRCs). We found that following KI many of the pLRCs proliferated and occasionally located in other parts of the kidney, suggesting their involvement in organ regeneration. We thus postulated that the kidney papilla is a niche for progenitor/stem cells. However, as pLRCs divide, the S-phase label marking them dilutes into their daughter cells, and their identification has remained elusive. Genetic lineage tracing of the pLRCs would allow this, but a specific marker was lacking. To obtain such a marker, we isolated live pLRCs and appropriate control cells from the H2B-GFP mouse and analyzed their transcriptional profile. After selecting a small number of genes overexpressed in the pLRCs, we performed qPCR analysis of the candidate genes and immunolocalized their encoded proteins in the kidney. Among these, protrudin, a cytoplasmic protein encoded by *Zfyve27*, identified a population of pLRCs. We then used it to perform lineage analysis of these cells by generating *Zfyve27-CreERT2* transgenic mice. Our results show that in the adult kidney *Zfyve27-CreERT2*-marked cells preferentially located in the upper part of the papilla. These cells do not generate progeny during homeostasis and thus appear not to contribute to normal kidney maintenance. However, after KI, particularly if severe, these cells are activated and follow a complex program of proliferation, migration, and morphogenesis generating multiple and long tubular segments located preferentially in the kidney medulla, indicating a critical role in the repair of this region of the kidney. Our results thus suggest that different parts of the kidney have different progenitor cell pools.

RESULTS

Isolation of Live pLRCs and Transcriptome Analysis

To search for potential markers of pLRCs, we administered doxycycline to H2B-GFP mice during embryonic life and chased them for 2–3 months, at which time GFP⁺ (i.e., LRCs) are essentially only found in the kidney papilla (Oliver et al., 2004, 2009). Single-cell suspensions of kidney papillae were subjected to fluorescence-activated cell sorting, and live GFP⁺ cells (i.e., pLRCs) and GFP⁻ control cells harvested for RNA extraction. In five independent experiments (each using both kidney papillae of nine H2B-GFP mice), mRNA from the live cells was amplified and analyzed by microarray. The results were deposited in the GEO website where they can be accessed (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71693>). As detailed in Supplemental Experimental Procedures, 216 transcripts were consistently overexpressed by >5-fold in the pLRCs versus GFP⁻ cells.

We confirmed the overexpression of the 30 highest-expressing genes in the GFP⁺ versus GFP⁻ cells using qPCR (Table S1). After consulting the databases of the Human Protein Atlas and Genepaint, we used antibodies for the proteins encoded by 19 of these genes (Table S2) and were able to detect 12 in the papilla. However, only one protein, protrudin (encoded by *Zfyve27*), located only in the papilla (and very rarely elsewhere in the kidney) (Figure 1A). In the H2B-GFP mouse, out of 1,008 GFP⁺ cells 10% were positive for protrudin (Figure 1B), demonstrating that the cells expressing this protein are a subpopulation of pLRCs. Protrudin appeared to be a cytoplasmic protein similar to what was found previously (Shirane and Nakayama, 2006). There are only a few published studies on this protein, but it has been implicated in the control of vesicular traffic and neuronal protrusions (Shirane and Nakayama, 2006; Matsuzaki et al., 2011).

Zfyve27-CreERT2-Marked Cells in the Kidney

pLRCs are by definition low cycling, but they actively proliferate and generate new cells after KI (Oliver et al., 2004, 2009). However, proliferation of LRCs dilutes the retained S-phase label, preventing identification of their progeny. To perform genetic lineage-tracing analysis of pLRCs, we generated BAC transgenic *Zfyve27-CreERT2* mouse lines driven by the protrudin promoter. We mated these mice with Reporter-tdTomato (*R-tdTomato*) mice and generated bitransgenic *Zfyve27-CreERT2;R-tdTomato* mice. We examined their kidneys for the presence of spontaneous tdTomato-expressing cells in 2-week-old, 2-month-old, and 6-month-old mice (n = 3 at each age) and found no tdTomato⁺ cells (not shown); thus, there was no Cre “leakage” in the absence of tamoxifen. In contrast, when *Zfyve27-CreERT2;R-tdTomato* adult mice were given

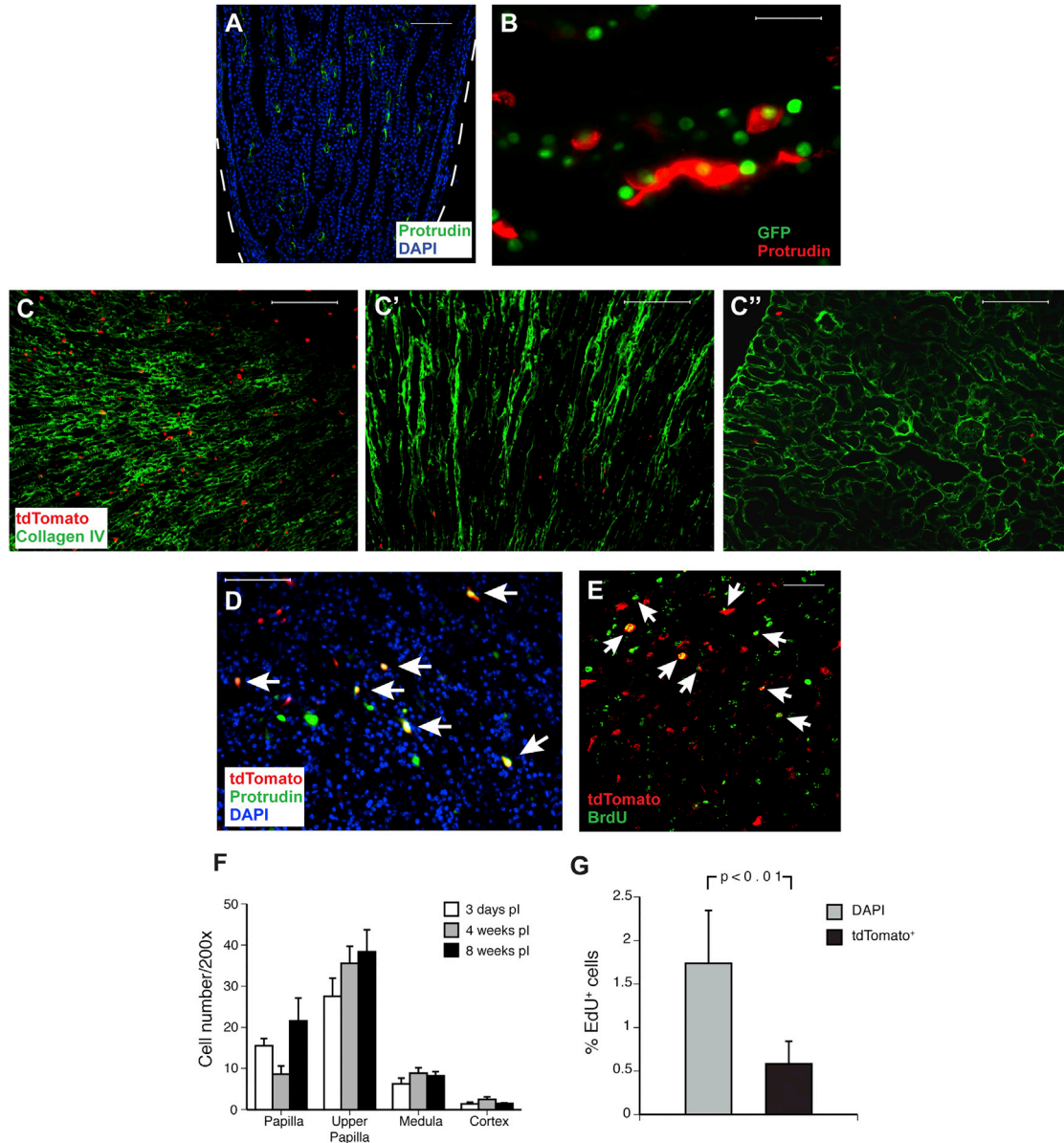


Figure 1. Kidney protrudin and *Zfyve27-CreERT2*-Marked Cells in Homeostasis

- (A) Mouse kidney papilla with scattered cells positive for protrudin. The dashed line shows the edge of the papilla. Scale bar, 100 μ m.
- (B) Protrudin was detected in \sim 10% of pLRCs ($n = 3$ mice). Scale bar, 20 μ m.
- (C–C'') *Zfyve27-CreERT2*-marked cells in the upper papilla (C), medulla (C'), and cortex (C'') 2 weeks pTM. Scale bars, 100 μ m.
- (D) Protrudin was detected (arrows) in many of the *Zfyve27-CreERT2*-marked cells in the upper papilla 1 day pTM ($n = 3$ mice). Scale bar, 50 μ m.
- (E) About 8% of *Zfyve27-CreERT2*-marked cells in the papilla 3 days pTM were BrdU-retaining (arrows); i.e., pLRCs ($n = 3$ mice). Scale bar, 40 μ m.
- (F) *Zfyve27-CreERT2*-marked cells in different kidney regions 3 days ($n = 10$), 4 weeks ($n = 6$) and 8 weeks ($n = 5$) pTM. The number of cells did not significantly increase from those at 3 days pTM. Mean \pm SE.
- (G) The fraction of tdTomato⁺ cells that incorporated EdU was significantly higher ($p < 0.01$) than that of the *Zfyve27-CreERT2*-marked cells. $n = 3$ mice 3 weeks pTM. Mean \pm SE.



tamoxifen and examined 2 weeks post tamoxifen (pTM), a small population of scattered tdTomato⁺ cells was detected in the kidney (see Figures 1C–1C''). Like pLRCs, *Zfyve27-CreERT2*-marked cells were most abundant in the upper part of the papilla (Figure 1C), which contained ~50% of all marked cells in the kidney while the rest of the papilla contained 33% of the cells. There were also scattered *Zfyve27-CreERT2*-marked cells in the medulla (13% of all labeled cells, Figure 1C') and the cortex (4% of all labeled cells, Figure 1C''), albeit at much lower densities.

With immunofluorescence, protrudin was detected (Figure 1D, arrows) in 39% of 134 *Zfyve27-Cre*-marked cells in the upper papilla and papilla. In contrast, no *Zfyve27-Cre*-marked cells in the cortex and medulla were found to be positive for protrudin. Furthermore, of 576 bromodeoxyuridine (BrdU)-retaining papillary cells, 8% were tdTomato⁺; i.e., marked by *Zfyve27-Cre*. Hence, *Zfyve27-CreERT2*-marked cells in the kidney papilla express protrudin and are a subpopulation of the pLRCs.

Like pLRCs (Oliver et al., 2004, 2009), the majority of *Zfyve27-CreERT2*-marked cells in the papilla appeared to be interstitial cells since they were present between collagen IV-positive basement membranes. However, also like pLRCs, a fraction of the *Zfyve27-CreERT2*-marked cells, averaging 25% (97 of 383 cells), were in collecting ducts (i.e., positive for AQP2; Figure S1A). Antibodies to AQP1 (identifying some thin descending limbs of Henle's loop) and to CLC-K1 (identifying thin ascending limbs) showed that few papillary *Zfyve27-CreERT2*-marked cells could be unambiguously located into these nephron segments, but the immunofluorescence signal of the thin segments of Henle's loop was weak. To obtain solid evidence for these structures, we used the more robust staining for protrudin in bitransgenic *Six2-Cre;R-tdTomato* mice (Humphreys et al., 2008) where epithelia of the papillary thin limbs of Henle's loop were easily identified by expressing tdTomato. We found (Figure S1B) that out of 4,269 tdTomato⁺ cells in the papilla, 14% were positive for protrudin.

Thus, in the kidney papilla, protrudin is expressed in interstitial cells, collecting duct cells, and thin limbs of Henle's loop. In previous studies we located pLRCs in the interstitial and collecting duct cell compartments (Oliver et al., 2004, 2009) and when we now examined *Six2Cre;R-tdTomato* adult mice, we also found BrdU-retaining (i.e., pLRCs) cells in the thin limbs of Henle's loop of the papilla (see Figure S1C).

Additional analysis of the papillary *Zfyve27-CreERT2*-marked cells with antibodies giving a robust (i.e., control) signal showed that these cells were negative for CD140b and CD146 (markers of pericytes/mesenchymal stem cells), CD68 (macrophages), CD11c (dendritic cells), p75NGFR (neural crest-derived cells) and α -smooth muscle actin. Recently, a sparse population of kidney cells express-

ing Sox9⁺ and generating progeny after injury was described in the kidney cortex and medulla (Kumar et al., 2015), but we found that *Zfyve27-CreERT2*-marked cells did not stain for SOX9 in these regions. However, a small fraction of the *Zfyve27-CreERT2*-marked cells in the upper papilla (17%) were Sox9⁺ (not shown). Finally, we characterized the papillary *Zfyve27-CreERT2*-marked cells using flow cytometry. We found (Table S3) that many tdTomato⁺ were positive for the cell-surface markers CD24, CD133, ITGB1(CD29), and ITGA6(CD49f) as well as SCA1, CXCR4, and CXCR7, albeit, surprisingly, substantial numbers of all other papillary cells were also positive for these markers. As shown, cell selection by combining CXCR4 and CXCR7 expression provided the sharpest distinction between *Zfyve27-CreERT2*-marked and non-marked papillary cells.

In the medulla and cortex, most of the *Zfyve27-CreERT2*-marked cells were interstitial and did not express any segment-specific markers. There were very few cells that could be identified as intratubular epithelial cells and some of them did express segment-specific markers. Of 553 cells, 18 were inside collagen IV basement membranes, two of which were megalin⁺, seven Tamm Horsfall protein⁺ (THP), and two calbindin⁺ (n = 6 mice). Thus, the marked cells were rarely present in differentiated epithelial structures and when there about half of them expressed the characteristic marker of that segment.

In summary, the *Zfyve27-CreERT2*-marked cells in the papilla are a subpopulation of pLRCs that express several markers, suggesting a role as progenitor cells in adult kidney.

***Zfyve27-CreERT2*-Marked Cells during Homeostasis**

In many organs, precursor/stem cells generate new cells only during homeostasis or only after organ injury or, at least in principle, under both conditions. To determine whether *Zfyve27-CreERT2*-marked cells contributed to organ maintenance in homeostasis, we quantified the number of tdTomato⁺ cells in all areas of the kidney at different times following administration of tamoxifen (Figure 1F); as shown, the number of marked cells did not significantly increase from 3 days to 8 weeks pTM in any of the kidney regions.

In *Zfyve27-CreERT2;R-tdTomato*-marked cells with tdTomato at clonal (Romagnani et al., 2015) or near clonal density (of 1,193 cells only ten were not present as single cells; see Table S4), we examined kidneys for the presence of possible tracing events (i.e., contiguous groups of >5 tdTomato⁺ cells derived presumably from a single-cell clone) at different times after tamoxifen. No tracing events were found in mice (n = 6) up to 4 months pTM. In mice analyzed up to 9 months pTM, very rare tracing events of marked cells were detected in three mice (total n = 6),



particularly in the medulla (Figure S2A), where sometimes tubules made up of marked cells were found (Figure S2A'). Only very rarely were tracing events detected in the cortex, as shown in the example of Figure S2A''. No tracing events were seen in the papilla.

Since one possible reason for the absence of tracing events is the large volume of the kidney and its low proliferation rate, we increased the number of marked cells by injecting the same dose of tamoxifen for five consecutive days. This resulted in an ~3- to 4-fold increase in the number of *Zfyve27-CreERT2*-marked cells (Figure S2B) in all regions of the kidney, and again the upper papilla contained most cells (Figure S2C). However, similar to the findings with 1 day of tamoxifen administration, the number of marked cells did not increase when the time after the drug was prolonged to 6 months (Figure S2B). In addition, in mice examined 6–9 months pTM ($n = 4$), we found no noticeable increase in tracing events from that observed after 1-day tamoxifen, suggesting that the initial number of marked cells had little effect on the frequency of detectable tracing events.

While these findings suggest that in homeostasis *Zfyve27-CreERT2*-marked cells very rarely proliferate, cell cycling in the adult kidney is normally very low and the cycling frequency of the marked cells could be that expected of all kidney cells. To test this, we administered ethyldeoxyuridine (EdU) to *Zfyve27-CreERT2*;R-tdTomato mice for 2 weeks, and the number of EdU⁺ cells that expressed tdTomato (i.e., *Zfyve27-CreERT2*-marked) or were only detected by nuclear DAPI was quantified. As shown in Figure 1G, 1.74% of tdTomato-negative (tdTomato⁻) cells were positive for EdU while only 0.58% of the *Zfyve27-CreERT2*-marked cells (i.e., tdTomato⁺) were positive ($p < 0.01$). Interestingly this difference was equally apparent in the kidney cortex, medulla, and papilla regions (not shown), indicating that the *Zfyve27-CreERT2*-marked cells in all regions of the kidney cycle less frequently than their neighboring cells, further confirming their relationship to pLRCs.

Thus, in the normal kidney *Zfyve27-CreERT2*-marked cells cycle at lower frequency than most kidney cells and lineage-tracing analysis indicates that, except for very rare occasions, they do not contribute to the homeostasis of the organ.

Response of *Zfyve27-CreERT2*-Marked Cells to Kidney Injury

Recent studies in skin, lung, and intestinal regeneration have shown that some stem cell pools proliferate in homeostasis, while others proliferate in response to injury. Even more surprising is the recent demonstration that in response to severe injury, specific stem cell populations are activated which differ from those that proliferate after

only mild injury (Tian et al., 2011; Vaughan et al., 2015; Chen et al., 2015; Tarlow et al., 2014). Stimulated by these studies, we examined the response of the *Zfyve27-CreERT2*-marked cells to graded KI by occluding the left renal artery for 10, 20, 30, and 40 min, and 8 days later tracing events were counted. In mice with KI due to 10 min or 20 min of artery occlusion ($n = 3$ for both times), no tracing events were detected 8 days after KI (not shown).

Following 30 min of ischemia, tracing events were easily detected in all injured kidneys, predominantly in the medulla. The tracing events consisted of many contiguous tdTomato⁺ cells, when examined transversally with multiple cuts by confocal microscopy (Figures 2A and 2A'), or tubular segments only containing tdTomato⁺ cells (Figure 2B). These results indicate repopulation of tubules by the tdTomato⁺ cells during kidney regeneration; i.e., kidney repair launches a tubulogenesis program by *Zfyve27-CreERT2*-marked cells. Most of these tubules were found in the medulla, with some in the cortex and none in the papilla. However, while the tubules with tdTomato⁺ cells were easily detected, they were isolated and their abundance was sparse (~1–3 detected per full sagittal kidney section), particularly in kidneys of mice treated with a single tamoxifen dose. However, in kidneys subjected to 40 min of renal artery occlusion we found a marked increase in the incidence of new tubules made up of *Zfyve27-CreERT2*-marked cells (see quantification below), particularly in the kidney medulla. Figure 2C shows the medulla of an injured kidney 8 days after KI while Figure 2C' shows the medulla of the contralateral normal kidney. When examined longitudinally, the length of the tubules containing multiple contiguous tdTomato⁺ cells varied from ~30 μm to more than 200 μm ; a graphic analysis of the lengths of 253 tubules is shown in Figure 2D where the median length was 62 μm ($n = 5$ mice). Occasionally, some of the tubules were very long, as shown in the ~235- μm tubular segment in Figure 2D'. The tubules preferentially located in the lower part of the medulla, the region contiguous to the papilla that contains abundant thick segments of Henle's loop, as shown in Figure 2E.

In sum, after severe KI, *Zfyve27-CreERT2*-marked cells at clonal (or near clonal) density generated multiple tubular structures in the kidney medulla, suggesting that they are precursor cells involved in repair of this part of the kidney. Remarkably, only severe injury led to full activation of this pool of progenitor cells, similar to what was seen in the skin and lung (Vaughan et al., 2015; Chen et al., 2015).

Proliferation of *Zfyve27-CreERT2*-Marked Cells during Kidney Repair

The generation of tubular structures after renal artery occlusion by *Zfyve27-CreERT2*-marked cells indicates that

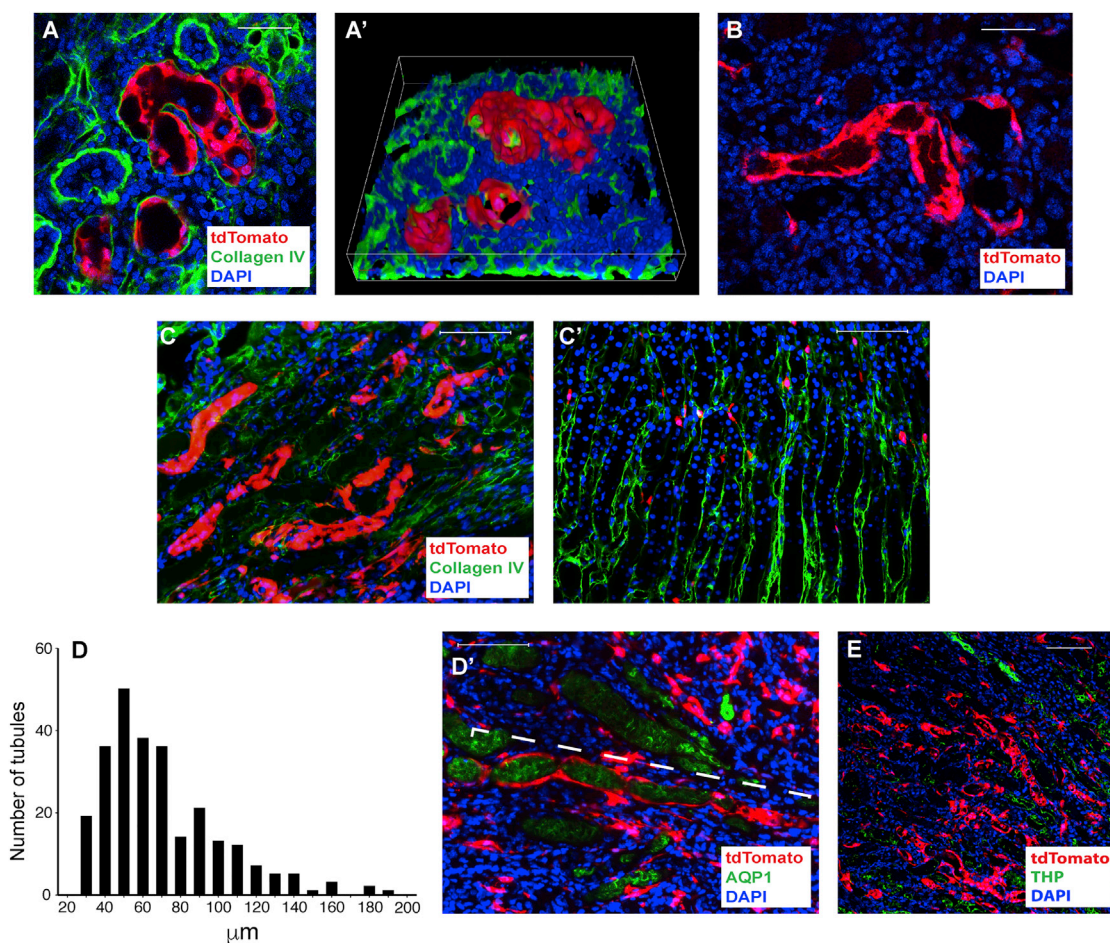


Figure 2. *Zfyve27-CreERT2*-Marked Cells during Kidney Repair from Injury

- (A) Kidney medulla of mouse with 30 min KI 14 days pTM. Scale bar, 25 μ m.
- (A') 0.3- μ m confocal cut of kidney medulla with yz and xz stacks 5 days after KI.
- (B) Kidney medulla of mouse with 30 min KI 3 days pTM. Longitudinal section of a medullary tubule made up of *Zfyve27-CreERT2*-marked cells 8 days after KI. Scale bar, 25 μ m.
- (C and C') Kidney medullae of mouse subjected to 40 min KI 8 days pTM in the injured kidney (C) and contralateral non-injured kidney (C') 8 days after KI. Scale bar, 50 μ m.
- (D) Bar graph of tubular length (μ m) of 253 tubules measured in the kidney medulla 8 days after 40 min KI in mice 8 days pTM (n = 5 mice).
- (D') Long tubule of *Zfyve27-CreERT2*-marked cells (dashed white line = 235 μ m) with a cast positive for AQP1 within it after 40 min KI 8 days pTM. Scale bar, 50 μ m.
- (E) The lower part of the medulla, with multiple thick ascending limbs of Henle's loop (positive for Tamm Horsfall Protein [THP]) contained the most tubules made by *Zfyve27-CreERT2*-marked cells; KI in mouse 8 days pTM. Scale bar, 100 μ m.

the cells actively proliferate during kidney repair. However, the extensive apoptosis and necrosis caused by renal artery occlusion is followed by widespread proliferation of kidney cells that reaches its maximum 2–4 days post injury (Forbes et al., 2000). To examine whether *Zfyve27-CreERT2*-marked cells cycle similarly to other kidney cells, we induced renal artery occlusion to *Zfyve27-CreERT2*;R-tdTomato mice and administered EdU at 3, 8, and 21 days after KI and quantified the number of EdU⁺ cell nuclei in both tdTomato⁺ cells (i.e., *Zfyve27-CreERT2*-marked) and in cells solely identified

by their DAPI nuclear staining (i.e., tdTomato⁻ cells) in the injured kidneys.

As shown in Figure 3A and illustrated in Figure 3B, 3 days after KI, a time at which cellular proliferation is most marked (Forbes et al., 2000), a similar fraction of the tdTomato⁺ and tdTomato⁻ cells incorporated EdU (~15%), indicating that the *Zfyve27-CreERT2*-marked cells cycled as other kidney cells. However, at 8 days and 21 days after KI, cycling by the tdTomato⁻ cells progressively declined while cycling by the tdTomato⁺ cells

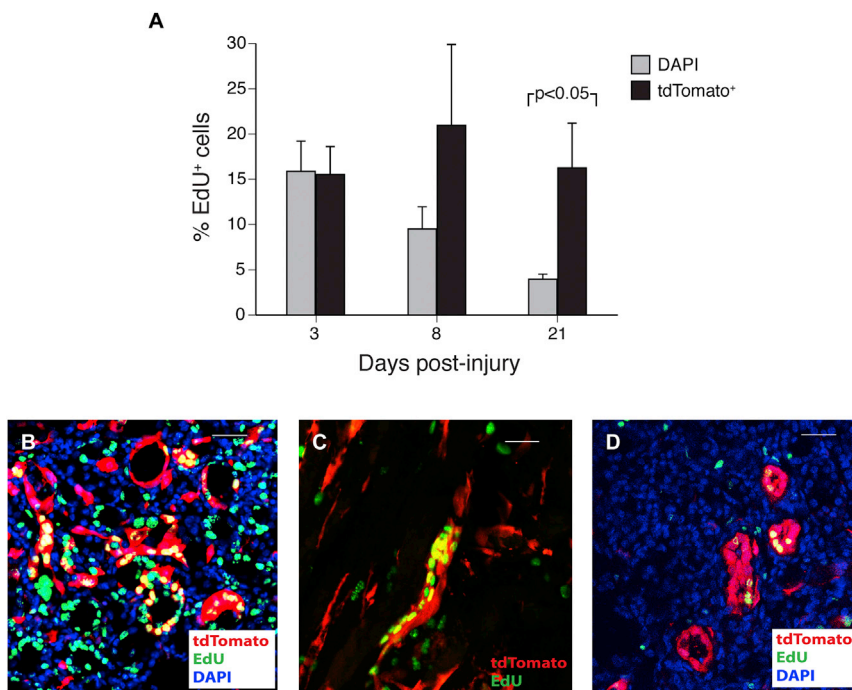


Figure 3. Proliferation of *Zfyve27-CreERT2*-Marked Cells during Kidney Repair from Injury

(A) Fraction (%) of tdTomato⁻ cells (identified with DAPI) and of tdTomato⁺ cells (i.e., *Zfyve27-CreERT2*-marked cells) that incorporated EdU at 3, 8, and 21 days after KI. EdU incorporation in tdTomato⁻ cells 21 days after KI was significantly lower than at 3 and 8 days ($p < 0.05$) and, as shown in the figure, than that of the tdTomato⁺ cells at this time ($p < 0.05$). $n = 3-5$ mice, 8 days pTM. Mean \pm SE.

(B) EdU incorporation in the upper papilla of a *Zfyve27-CreERT2*;R-tdTomato mouse 3 days after KI. Scale bar, 40 μ m.

(C) Cluster of tdTomato⁺ cells in the upper papilla that incorporated EdU 8 days after KI. Scale bar, 40 μ m.

(D) Tubules made up of tdTomato⁺ cells that incorporated EdU 21 days after KI. Scale bar, 50 μ m.

continued unabated (Figure 3A). At this time, we occasionally observed groups of proliferating tdTomato⁺ cells in the upper papilla (Figure 3C), as we previously had seen with pLRCs in the H2B-GFP mouse (Oliver et al., 2009). As shown 21 days after KI in Figure 3A and illustrated in Figure 3D, proliferation by tdTomato⁺ cells continued unchanged while cycling by tdTomato⁻ cells had markedly decreased and approached, at this time, that of cells in normal kidneys (see Figure 1G). Indeed, the fraction of tdTomato⁻ cells that incorporated EdU at 21 days after KI was significantly lower than at 3 ($p < 0.01$) and 8 days ($p < 0.05$) and that of the tdTomato⁺ cells at this time ($p < 0.05$). Thus unlike unmarked cells, *Zfyve27-CreERT2*-marked cells have long-term proliferating potential.

Tubular Segments Regenerated from *Zfyve27-CreERT2*-Marked Cells Are Mostly in the Medulla

Tracing events generated from single-cell clones were recently described in the kidney during both homeostasis and after injury by Rinkevich et al. (2014), who used the ubiquitous actin promoter to conditionally activate Cre in the adult kidney of bitransgenic mice and randomly generated single-cell clones expressing a reporter protein. Long-term observation of normal kidneys or with KI revealed formation of nephron tubular segments made of clone-restricted cells. Since the tubules were restricted to specific nephron segments, these results suggested the presence of fate-restricted precursor cells in different parts of the nephron.

Thus, as with the actin-Cre construct, *Zfyve27-CreERT2* might have stochastically marked fate-restricted precursor cells and their proliferation after KI generated the new tubules made of *Zfyve27-CreERT2*-marked cells. To test this, we designed parallel experiments with mice that are likely to express tdTomato in kidney cells in a random manner. We used the *Rosa26-CreERT2* mouse, which in the *Rosa26* locus contains CreERT2, to generate bitransgenic *Rosa26-CreERT2*;R-tdTomato. For comparison of the response to KI of *Zfyve27-CreERT2*;R-tdTomato mice with that of the *Rosa26-CreERT2*;R-tdTomato, the number of tdTomato⁺ cells had to be similar in both mouse lines, but administration of a low dose of tamoxifen (40 mg/kg) to *Rosa26-CreERT2*;R-tdTomato labeled all kidney cells (not shown). By progressively decreasing the tamoxifen dose to 5 mg/kg, as shown in Figure 4A, in *Rosa26-CreERT2*;R-tdTomato we marked comparable numbers of tdTomato⁺ cells with those of *Zfyve27-CreERT2*;R-tdTomato mice. We then induced 40 min of left renal artery occlusion to both *Zfyve27-CreERT2*;R-tdTomato mice and *Rosa26-CreERT2*;R-tdTomato mice and harvested both the injured and contralateral, non-injured kidneys at 8 days after KI. Clonal analysis of the tdTomato cells present in the control kidney for both groups of mice (Figure S3B) showed that the vast majority of the cells were single clones (97% in *Zfyve27-CreERT2*;R-tdTomato and 93% in *Rosa26-CreERT2*;R-tdTomato mice). In contrast, the injured kidneys in both mouse strains showed multiple tubules that contained either ten or more contiguous marked cells when

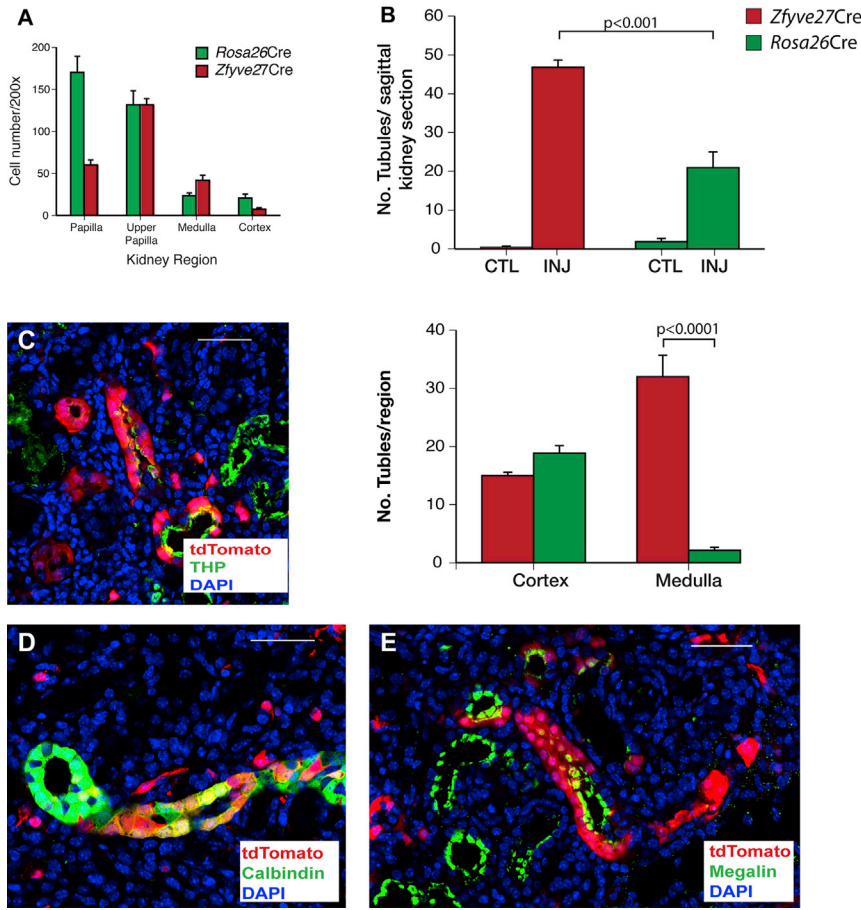


Figure 4. Specificity of the New Tubular Segments Generated by *Zfyve27-CreERT2*-Marked Cells during Kidney Repair

(A) Number of tdTomato⁺ cells in *Zfyve27-CreERT2*;R-tdTomato (n = 8) and *Rosa26-CreERT2*;R-tdTomato (n = 7) mice 8 days pTM. In the upper papilla, medulla, and cortex both groups had similar cell numbers but in the rest of the papilla, *Rosa26-CreERT2*;R-tdTomato mice had a much greater number (p < 0.001). Mean ± SE. (B) Tubules made up of tdTomato cells in injured kidneys (INJ) of *Zfyve27-CreERT2*;R-tdTomato mice (n = 5; in red) and *Rosa26-CreERT2*;R-tdTomato mice (n = 6; in green) 8 days after KI, performed 8 days pTM. The average number of tubules per kidney section is shown in the top. Since independently of injury tamoxifen occasionally generated tubules in *Rosa26-CreERT2*;R-tdTomato mice (Figure S3B'), quantification of the tubules in both the injured (INJ) and non-injured kidneys (CTL, control) is shown. In both mice, KI induced generation of tubules made up of tdTomato⁺ cells (p < 0.0001 versus the control kidney) but the number of tubules in the injured kidney of the *Zfyve27-CreERT2*;R-tdTomato mice was significantly greater than that in the *Rosa26-CreERT2*;R-tdTomato mice (p < 0.001). Mean ± SE. The regional distribution of the tubules made up tdTomato⁺ cells

is shown in the bottom panel. In the cortex, the number of new tubules was very similar for both groups of mice. In contrast, while the medulla of the *Zfyve27-CreERT2*;R-tdTomato mice contained 68% of the total number of new kidney tubules, the medulla of the *Rosa26-CreERT2*;R-tdTomato mice had very few (12%). Mean ± SE.

- (C) Tubular structures made up of *Zfyve27-CreERT2*-marked cells expressing Tamm Horsfall protein (THP). Scale bar, 50 μm.
 (D) Tubular structure made up of *Zfyve27-CreERT2*-marked cells expressing calbindin. Scale bar, 40 μm.
 (E) Tubular structures made up of *Zfyve27-CreERT2*-marked cells expressing megalin. Scale bar, 50 μm.

sectioned longitudinally or only tdTomato⁺ if sectioned transversally; their numbers are shown in Figure 4B (top). There were twice as many tubules generated in the *Zfyve27-CreERT2* mice (p < 0.01) despite a similar abundance of tdTomato-marked cells before injury. More noteworthy however, was the strikingly different location of the new tubules made up of tdTomato⁺ cells, as shown in the lower panel of Figure 4B. In *Rosa26-CreERT2*;R-tdTomato mice almost all marked tubules were found in the kidney cortex (88%) and a small number (12%) in the medulla. In contrast, in *Zfyve27-CreERT2*;R-tdTomato mice most of the tubules of tdTomato⁺ cells were found in the medulla (68%) while the cortex contained 32%.

To exclude the possibility that ischemic KI might activate *Zfyve27* and that the presence of residual tamoxifen 8 days pTM could account for our findings, we performed KI in *Zfyve27-CreERT2*;R-tdTomato mice at 4 and 8 weeks

pTM and examined their kidneys 8 days after KI. In mice 4 weeks pTM (n = 3), we found an average of 60 tubules per sagittal kidney section (76% of them located in the medulla) and in mice 8 weeks pTM (n = 4) we found 55 tubules per sagittal kidney section (69% of which located in the medulla), in agreement with the data of Figure 4B.

A simple model of the data from Figures 4A and 4B would predict that if all single labeled cells can generate a clone of cells after injury, it would be expected that the number of labeled tubules in the injured kidneys would be similar to that of labeled cells in control kidneys. Indeed, in the kidney cortex the number of labeled tubules was similar to the number of individual cells in both the *Rosa26-Cre* mice (21 cells in control kidneys and 19 tubules in injured kidneys) and *Zfyve27-Cre* mice (7 cells and 15 tubules). In the medulla, however, the control *Rosa26-Cre* mice



Table 1. Fraction of Tubules Made of tdTomato⁺ Cells with Nephron-Segment-Specific Markers after KI in *Zfyve27-CreERT2;R-tdTomato* and *Rosa26-CreERT2;R-tdTomato* Mice

	8 Days after KI (%)		4 Weeks after KI (%)	
	Medulla	Cortex	Medulla	Cortex
<i>Zfyve27-CreERT2;R-tdTomato</i>				
Megalin	0	30	0	28
THP	12	0	21	9
Calbindin	11	0	8	26
AQP2	0	5	0	0
<i>Rosa26-CreERT2;R-tdTomato</i>				
Megalin	0	45	0	80
THP	0	0	0	0.2
Calbindin	0	0	0	0
AQP2	0	0	0	3

KI was performed 8 days pTM. *Zfyve27-CreERT2;R-tdTomato* mice n = 5 at 8 days and n = 6 at 4 weeks. *Rosa26-CreERT2;R-tdTomato* mice n = 6 at 8 days and n = 5 at 4 weeks.

had 23 cells while *Zfyve27-Cre* mice had 42 cells. Yet the number of labeled tubules in the injured kidney medulla of *Rosa26-Cre* mice was only 2, while that of the *Zfyve27-Cre* mice was 32. Thus it appears that in the medulla, single cells labeled by *Zfyve27-Cre* led to the appearance of tubules after injury while the randomly labeled *Rosa26-Cre* cells rarely did. This suggests that the *Zfyve27-CreERT2*-marked cells are specific precursors of cells needed for repair of the kidney medulla after severe KI.

Differentiation of the Progeny of *Zfyve27-CreERT2*-Marked Cells

To identify the progeny generated by *Zfyve27-CreERT2*-marked cells after KI, we used nephron segment-specific cell markers. We probed kidney sections with antibodies to megalin (proximal tubule), THP (thick ascending limb of the loop of Henle), calbindin (distal tubule), and AQP2 (collecting duct). Although the kidney medulla also contains abundant thin ascending and descending loops of Henle, using several antibodies these nephron segments could not be consistently identified after KI and were excluded from analysis.

Eight days after KI injury, we found that some medullary tubules made of *Zfyve27-CreERT2*-marked cells were positive for THP (see example in Figure 4C) or calbindin (see Figure 4D). However, for THP, of 66 tubules examined only 8 (12%; see Table 1) were positive, and of 57 examined for calbindin, only 6 (11%) were positive. No tubule in the medulla was positive for megalin or AQP2 and, thus, the

majority of the tubules were unidentified. Four weeks after KI, the number of tdTomato⁺ tubules in the medulla identified as thick ascending limbs of Henle's loop (i.e., THP positive) had increased (Table 1) to 21% (16 of 76), indicating that expression of THP lags behind tubulogenesis during kidney repair. The frequency of tubules made up of *Zfyve27-CreERT2*-marked cells that were positive for calbindin did not significantly change (8%; 5 of 59) at 4 weeks. Thus, even at this late date, the majority of the new medullary tubules made of *Zfyve27-CreERT2*-marked cells did not express the terminally differentiated markers of these segments.

Interestingly, even though no tubule was identified as a proximal tubule in the medulla, a third of tdTomato⁺ tubules in the cortex (see Table 1) were positive for megalin (see example in Figure 4E), both at 8 days (31%; 17 of 54) and 4 weeks after KI (28%; 5 of 18), suggesting that *Zfyve27-CreERT2*-marked cells in the medulla and cortex generate different progeny. In addition, 4 weeks after KI injury, 9% (4 of 46) of the cortical tubules were positive for THP and 26% (6 of 23) were positive for calbindin. Thus, unlike the medulla, almost 70% of all cortical tubules made up of tdTomato⁺ cells could be identified 4 weeks after KI, with one third being proximal tubules, another third distal tubules, and ~10% thick ascending loops of Henle.

In *Zfyve27-CreERT2;R-tdTomato* mice in which KI was induced 4 weeks (n = 3) and 8 weeks (n = 4) pTM, similar results were found in both groups and the data were pooled. Eight days after KI, of an average of 15 cortical tubules per kidney sagittal section, 33% were positive for megalin and of an average of 42 medullary tubules, 6% were positive for THP and 14% for calbindin, results comparable with those of Table 1.

In marked distinction to the findings in *Zfyve27-CreERT2;R-tdTomato* mice, in the injured kidneys of the *Rosa26-CreERT2;tdTomato* mice the medulla very rarely had tubules (see Figure 4B, bottom), and in none expressed differentiation markers that allowed the segments to be identified. Most of the new tubules made up of tdTomato⁺ cells in these mice were restricted to the kidney cortex (Table 1) and 4 weeks after KI, 80% of them (24 of 30) were MEGALIN positive, identifying them as proximal tubules.

Thus, most cortical tubules after KI in *Rosa26-CreERT2;tdTomato* mice rapidly acquired their differentiation markers. However, recovery from injury in the medulla of *Zfyve27-CreERT2;R-tdTomato* was much slower, with only a small fraction of thick ascending and distal tubules acquiring their differentiated status. A possible reason for this is that these marked cells continue to proliferate for long periods of time (Figure 3A) and it is well known that differentiation is suppressed during cell division.

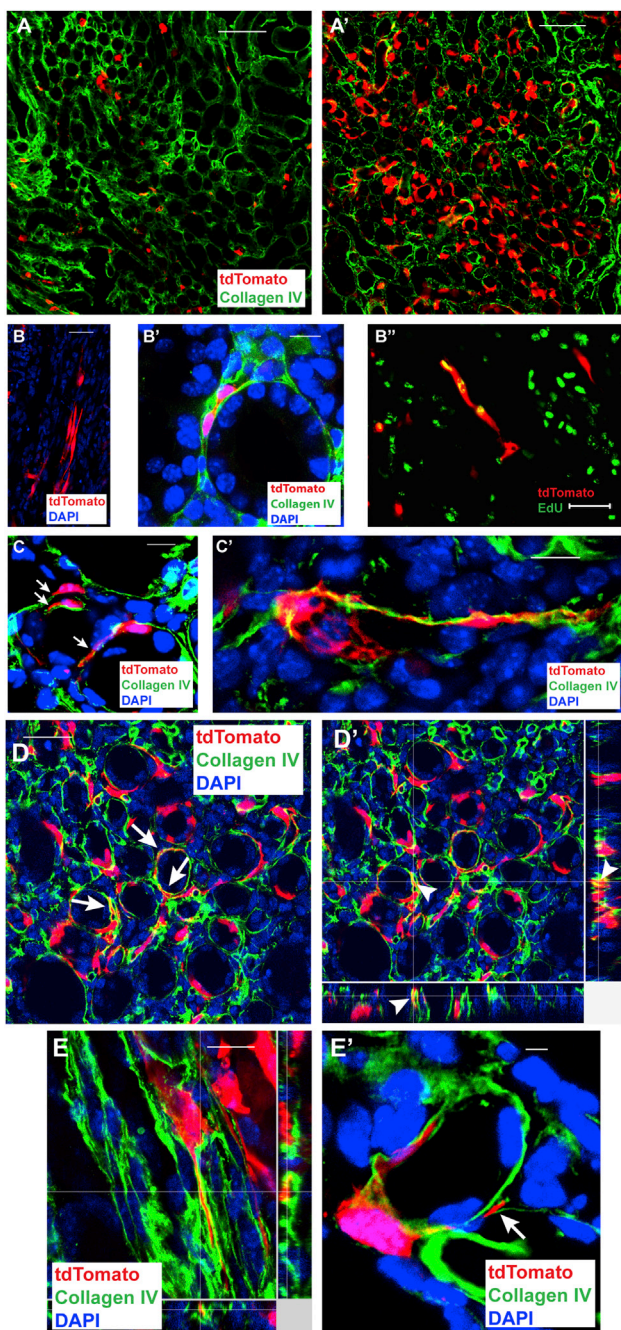


Figure 5. Migratory Phenotype of *Zfyve27-CreERT2*-Marked Cells during Kidney Repair

(A and A') Transverse sections of the upper papillae in control (A) and injured (A') kidneys of a *Zfyve27-CreERT2*;R-tdTomato mouse 3 days after KI, performed 10 days pTM. Scale bar, 40 μ m. (B–B'') High-power images of *Zfyve27-CreERT2*-marked cells with elongated bodies (B; scale bar, 30 μ m), nuclear deformation (B'; scale bar, 20 μ m), and association in chains (B''; scale bar, 25 μ m). (C and C') The *Zfyve27-CreERT2*-marked cells displayed broad projections like lamellipodia (arrow in C) and finger-like projections typical of filopodia (C'). Both scale bars, 20 μ m.

Migration of *Zfyve27-CreERT2*-Marked Cells after Kidney Injury

Repair of many epithelial organs involves, in addition to cell proliferation, a dynamic program of cell migration and differentiation (Blikslager et al., 2007; Veniaminova et al., 2013). Epithelial precursor cells actively migrate in *Drosophila* Malpighian tubules during homeostasis (Singh et al., 2007), and collective epithelial cell migration is an early response to injury in zebrafish kidney (Palmyre et al., 2014). Since we previously found that after KI some pLRCs migrated outside the kidney papilla (Oliver et al., 2009), we considered that since the majority of *Zfyve27-CreERT2*-marked cells were in the upper papilla, their migration toward the adjacent medulla could be the reason for preferential repair of these medullary nephron tubules.

In kidney sections of *Zfyve27-CreERT2*;R-tdTomato mice subjected to KI, we found that the *Zfyve27-CreERT2*-marked cells displayed multiple morphological characteristics of cell migration (Ridley et al., 2003). First of all, 3 days after injury, when compared with cells in the normal contralateral kidney, the tdTomato⁺ cells in the upper papilla of the injured kidney broadened their shape and rounded around tubular structures (Figures 5A and 5A'). At higher power, many of these marked cells had elongated bodies (Figure 5B), narrow nuclei (Figure 5B'), and frequently aggregated in chain-like structures (Figure 5B''). In addition, as in regeneration in other epithelia, multiple cells had prominent cellular protrusions, either with the broad shape characteristic of lamellipodia (Figure 5C) or finger-like filopodia (Figure 5C'). None of these shape changes were seen in the uninjured contralateral kidney.

Remarkably, many of the tdTomato⁺ cells were found to invade and traverse the collagen IV basement membranes, as shown in the confocal images of a transverse section of the kidney medulla (Figures 5D and 5D'). A high-power view of these cells revealed cellular protrusions that invaded the extracellular matrix (Figure 5E) or crossed the basement membrane of tubular structures (Figure 5E'). We suggest that the traversal of the basement

(D and D') Transverse section of the kidney medulla 8 days after KI, performed 10 days pTM. Confocal 0.3- μ m cut (D) and orthogonal projections (D') showing that cell extensions penetrated collagen IV basement membranes (arrows in D and arrowheads in D'; scale bar, 50 μ m).

(E) High-power confocal ortho-projection of a single *Zfyve27-CreERT2*-marked cell penetrating into the collagen IV matrix 8 days after KI performed 10 days pTM. Scale bar, 20 μ m.

(E') High-power confocal 0.3- μ m cut of a single *Zfyve27-CreERT2*-marked cell with projections inside and outside (arrow) a tubule collagen IV basement membrane 8 days after KI performed 9 days pTM. Scale bar, 20 μ m.

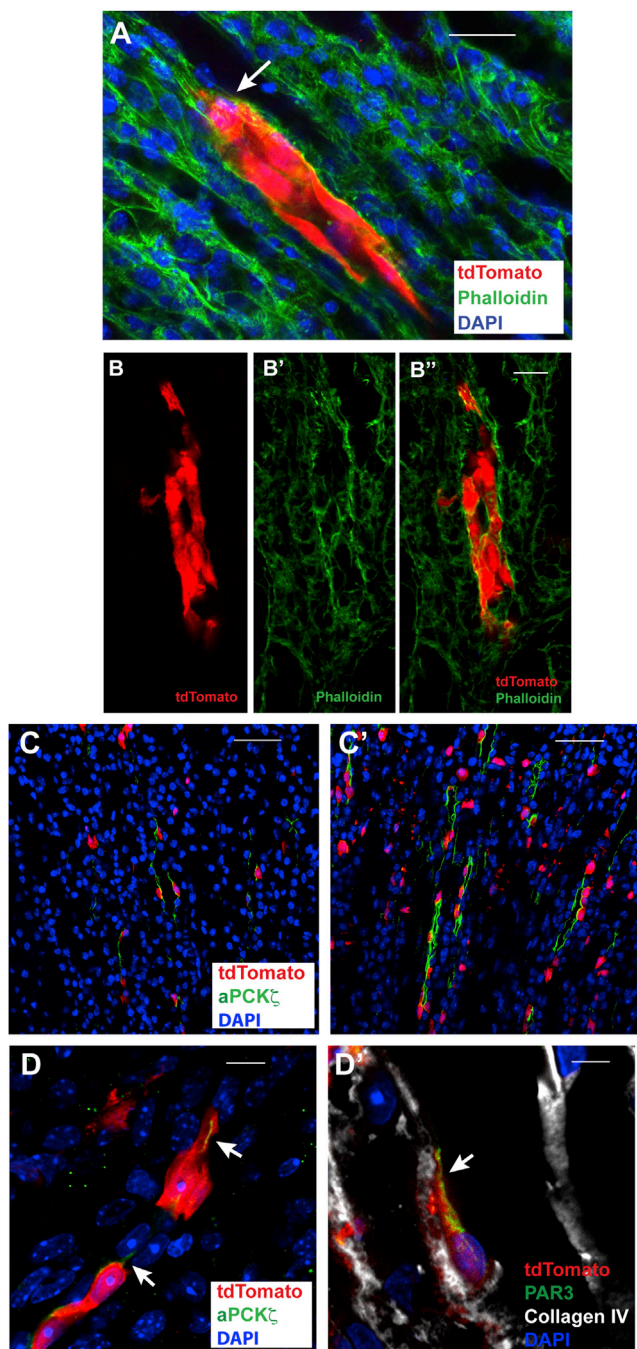


Figure 6. Expression of Polarity Proteins in *Zfyve27-CreERT2*-Marked Cells during Kidney Repair

(A) Groups of *Zfyve27-CreERT2*-marked cells with actin polymerization in the leading cells (arrow) 8 days after KI, performed 10 days pTM. Scale bar, 20 μ m.

(B–B'') Group of *Zfyve27-CreERT2*-marked cells with actin polymerization at the edges of the cell cluster 8 days after KI. Scale bar, 50 μ m. (C and C') aPKC ζ expression in the upper papilla of the control (C) and injured (C') kidneys 3 days after KI, performed 10 days pTM. Scale bar, 60 μ m.

membrane implies that these cells initially located in the interstitial might begin to populate injured medullary tubules.

Another striking change induced by KI was the appearance of aggregated clusters of *Zfyve27-CreERT2*-marked cells that exhibited actin polymerization either in the leading cells (Figure 6A) or at the edges of the cell cluster (Figures 6B–6B''), suggesting collective cell migration (Lucas et al., 2013; Xu et al., 2014), a process originally discovered during gastrulation but subsequently found in a variety of adult systems including mammary gland morphogenesis and epithelial organ repair (Friedl and Gilmour, 2009), including zebrafish kidney (Palmyre et al., 2014).

Finally, migrating epithelial cells become polarized, exhibiting an apical complex of aPKC ζ , PAR3, and PAR6, proteins which accumulate in the leading edges of cellular projections during cell migration (Shin et al., 2007). We found that 3 days after KI, aPKC ζ expression markedly increased in the papilla of the injured kidney (Figures 6C and 6C'). In addition, high-power confocal microscopy showed that expression of aPKC ζ (Figure 6D) and PAR3 was concentrated at the leading processes of tdTomato⁺ elongated cells (Figure 6D'). In non-injured kidney aPKC ζ was restricted to few *Zfyve27-CreERT2*-marked cells in the papilla. It is interesting to note that protrudin/*Zfyve27* has been associated with development of large protrusions in neurons and contains FYVE domains that bind phosphatidylinositol 3-phosphate molecules (Raiborg et al., 2015) similarly to CDC42, another FYVE domain-containing protein that is involved in polarized motility.

These studies demonstrate that after injury the *Zfyve27-Cre*-marked cells change shape, display cellular projections that invade extracellular matrix, aggregate, and become polarized, all consistent with cell migration, including collective cell migration as seen in other organs during response to injury. While it is likely that the new tubules containing tdTomato⁺ cells after kidney repair derive from the epithelial protrudin-expressing cells, these results raise the possibility that interstitial progenitor cells might invade the tubule and proliferate, replacing tubular epithelial cells. Indeed, integration of interstitial cells into nephron epithelia has previously been reported (Li et al., 2015), although more direct studies will be needed before this issue can be settled.

Migratory Capacity of *Zfyve27-CreERT2*-Marked Cells

As direct demonstration of cell migration in mammalian kidney in vivo is currently not possible, to test whether

(D and D') High-power confocal microphotographs of *Zfyve27-CreERT2*-marked cells 3 days after KI, performed 10 days pTM, expressing aPKC ζ (arrows in D; scale bar, 20 μ m) and PAR3 (arrow in D'; scale bar, 20 μ m) at their leading processes.



Zfyve27-CreERT2-marked cells in the upper papilla had migratory capacity, we examined their behavior during organ culture. One half of a kidney from *Zfyve27-CreERT2*; *R*-tdTomato mice was analyzed with a two-photon microscope whereby the tdTomato⁺ cells in the upper papilla were easily identified and the fluorescein isothiocyanate (FITC)-labeled papilla provided spatial information (see Figure S4A). In three different experiments, 233 *Zfyve27-CreERT2*-marked cells were detected, and 28 of these cells (12%) were found to be moving with an average speed of $46.6 \pm 4.0 \mu\text{m/h}$ (mean \pm SE). In one experiment, two tdTomato⁺ had velocities greater than $100 \mu\text{m/h}$ and, suspected of being circulating cells, were excluded from the analysis. Interestingly, almost three-fourths of the 28 moving cells were migrating away from the FITC-containing lower papilla and toward the medulla. An experiment is shown in the movie of Figure S4B.

DISCUSSION

The adult kidney has a low rate of homeostatic cell cycling; yet, following injury, even one severe enough to cause total renal failure, widespread cell proliferation and functional recovery can occur. The mechanisms responsible for generation of new kidney cells during homeostasis and during organ repair remain poorly understood. Our previous studies had suggested that pLRCs might function to replenish these damaged cells, but we sought to find a lineage marker to allow definitive tracing of the progeny of these cells. Here we found that *Zfyve27* (protrudin) is a marker of a subpopulation of the pLRCs and using *Zfyve27-CreERT2* lines, we found that marked cells were most abundant in the upper part of the kidney papilla but that other parts of the kidney also had cells, albeit at much lower numbers. These labeled (and protrudin-expressing) cells were themselves label-retaining and cycled at much lower rates than the rest of the renal cells. Remarkably, they did not seem to participate in cell replacement during homeostasis or even kidney repair after modest injury. Yet, during severe KI these cells generated clones that populated large segments of injured tubules and, even more surprisingly, these tubules seemed to be located specifically in the kidney medulla rather than the cortex. Following severe injury, these cells continued to cycle longer than the rest of the kidney, explaining perhaps the extensive replacement of medullary tubules by their progeny. Hence, these results demonstrate that in the kidney (as in other epithelial organs) there exist multiple pools of progenitor cells, each of which is responsible for specific and perhaps anatomically distinct roles.

We identified the *Zfyve27* marker based on isolation of pLRCs that expressed the chimeric protein H2B-GFP. This

protein has been found to be retained for a long time by cells that do not or seldom cycle and to dilute into the progeny of dividing cells (Brennand et al., 2007; Wilson et al., 2008; Foudi et al., 2009). Although these studies strongly suggested that H2B-GFP retention is a good marker for low-cycling cells, it is still theoretically possible that in the kidney papilla H2B-GFP is degraded at variable rates in different cells and that “H2B-GFP retention” is unrelated to cycling activity. However, in our previous studies we had found that the H2B-GFP-retaining cells of the kidney had the same location as those that retained BrdU, and they responded to injury in a qualitatively and quantitatively similar manner (Oliver et al., 2004, 2009).

The *Zfyve27*-marked cells in the papilla, as well as many other papillary cells (see Table S3), contain cell-surface markers found in a population of scattered proximal tubular cells (Smeets et al., 2013) implicated in kidney regeneration. However, because *Zfyve27*-marked cells in the cortex and medulla were very sparse, our flow cytometry analysis was restricted to papillary cells, and it is unknown whether cortical and medullary cells contain the same markers and are related to those in the papilla.

We had previously shown that after KI pLRCs migrated out of the upper papilla, and we show here that *Zfyve27-CreERT2*-marked cells assume a phenotype compatible with migratory behavior, even collective cell migration extending protrusions and filopodia in a polarized manner. Indeed, in ex vivo organ culture these marked cells were capable of moving at considerable speeds. Protrudin was initially identified as important in endocytosis, and it is well known that endocytosis is a critical mechanism in the formation of the leading edge during polarized motility (Bretscher, 2014). Moreover, recent studies have shown that protrudin (in neurons) binds to motor proteins, facilitating the movement of membrane to the surface to induce membrane extensions (Raiborg et al., 2015). While final proof of cell migration during repair of mammalian kidney in vivo would require additional studies with more invasive methods, we suggest that the *Zfyve27*-marked cells were identified (serendipitously) as those pLRCs that migrate in response to injury.

Generation of new cells during kidney repair has long been assumed to result from proliferation of surviving terminally differentiated cells. However, much of the modern evidence has used lineage tracing such as *Six2* (Humphreys et al., 2008) or a terminally differentiated marker (such as the proximal tubule phosphate transporter) (Kusaba et al., 2014). Such studies cannot eliminate the possibility that nephron segments might contain progenitor cells that express these markers or are derived from this lineage. Indeed, we found that some *Six2*-derived cells in the papilla expressed protrudin, as were some pLRCs, and together with *Zfyve27*-marked cells, protrudin-expressing cells (as well as



pLRCs) were found in all three currently known embryonic cell lineages present in papilla: interstitial as well as the two epithelial cells (*Six2*-derived and collecting ducts). Thus, each different pool of pLRCs (or *Zfyve27*-marked) cells might generate progeny restricted to their parent compartment; therefore the new tubules containing tdTomato⁺ cells found after kidney repair most likely derive from the epithelial protrudin-expressing cells.

This finding is compatible with results showing that during kidney repair *Six2*-marked cells showed no dilution of the marker after kidney regeneration (Humphreys et al., 2008). Moreover, recent studies by Rinkevich et al. (2014) using the actin promoter to generate random single-cell clones in the adult kidney showed that some marked cells with high proliferation capacity expanded and generated tracing events restricted to specific nephron segments, indicating that the adult kidney likely contains precursor cells that are segment specific. They also found that cells with high proliferation capacity were Wnt responsive, raising the question of whether the *Zfyve27-CreERT2*-labeled cells (likewise with high proliferative capacity after KI) might be the same population as the Wnt-responsive cells identified by Rinkevich et al. (2014), a line of inquiry that we plan to pursue in the future.

The full power of the *Zfyve27-CreERT2*-marked cells to regenerate tubules was observed only after severe KI, since milder injury known to induce apoptosis did not induce the cells to proliferate and there was only modest proliferation by the cells after 30 min of kidney ischemia, a potent stimulus for widespread cell cycling in the kidney (Forbes et al., 2000; Oliver et al., 2009). It is thus possible that recovery from modest injury depends on proliferation of another pool of progenitor cells that belongs to a different lineage. These might include the aforementioned segment-specific or other types of progenitors or even terminally differentiated cells, as occurs in the liver (Yanger et al., 2014). Severe injury might deplete these latter pools of progenitors, as was found elsewhere (Tian et al., 2011; Vaughan et al., 2015). The medullary restriction of the *Zfyve27-CreERT2*-labeled cells emphasizes that the kidney, like other organs (Page et al., 2013; Donati and Watt, 2015), appears to have several precursor cell populations to generate new cells. For instance, stem cells in different segments of an organ, such as the gut, possess cell-autonomous region specificity (Wang et al., 2015) and, while the vertebrate kidney is seen as an organ, it is made up of semi-autonomous units called nephrons (containing different cellular regions), which in more ancient animals are fully individual organs (Hazelton et al., 1988; Scimone et al., 2011).

Characterization of the *Zfyve27-CreERT2*-marked cells in the kidney remains to be completed, but work in other organs suggests several possibilities. They could be bona fide

precursor/stem cells that in addition to multilineage potential are able to self-renew and are solely activated during severe organ injury, but to prove multipotency and self-renewal will require analysis of individual cells inside their niche in vivo. Another possibility could be that if the kidney papilla is, as we previously suggested (Oliver et al., 2004), a precursor/stem cell niche, severe injury might have ablated resident stem cells and signals from the niche would then induce *Zfyve27-CreERT2*-marked (and other) cells to repopulate the niche and, by changing cell identity, become facultative stem cells, as found in regeneration of several organs (Tian et al., 2011; van Es et al., 2012).

EXPERIMENTAL PROCEDURES

Genome Expression Analysis of pLRCs

All animal experiments were performed under the oversight of the Institutional Animal Care and Use Committee of Columbia University.

To obtain the transcription profile of the pLRCs, we pulsed H2B-GFP mice with doxycycline during embryonic life and chased to ~2–3 months, as described by Oliver et al. (2009). In five different experiments, nine mice were euthanized and the kidney papillae isolated. Papillary cells were dispersed with collagenase digestion as described by Oliver et al. (2009). To label cells of bone marrow and endothelia origin, we briefly incubated cells with anti-mouse antibodies to CD45, TER119, and CD31, all coupled to PE (Becton Dickinson). In a BD FACScaria, PE-negative live cells (DAPI-negative) were sorted into GFP-positive and GFP-negative (~2,000 cells in each sample) populations directly into lysis buffer. After RNA extraction and labeling, samples were analyzed with Two-Color Agilent Whole Mouse Genome Microarrays (Miltenyi Biotec).

Generation of Mice to Label *Zfyve27*-Expressing Cells

To genetically mark, in a regulated manner, *Zfyve27*-expressing cells, we first inserted a CreERT2 cassette into the promoter of *Zfyve27*, and cloned an Frt-NEO-Frt (FNF) cassette downstream of the CreERT2 to amplify the CreERT2-FNF cassette by high-fidelity PCR (Invitrogen AccuPrime High-Fidelity Taq PCR kit) with primers harboring 50-bp short homologies from both sides of the *Zfyve27* ATG codon. This CreERT2-FNF cassette was then recombined into the *Zfyve27* ATG codon of a BAC-containing *Zfyve27* gene by BAC recombining method. A gene-targeting vector was then generated by retrieving a 2-kb short arm (5' to CreERT2-FNF insertion), CreERT2-FNF, and a 5-kb long arm (CreERT2-FNF to 3') onto a plasmid carrying diphtheria toxin α chain (DTA cassette) as negative selection during gene targeting. 10 μ g of pMCS-*Zfyve27-CreERT2-DTA* was linearized by *AscI* and electroporated into KV1 embryonic stem cells, and selected with 200 μ g/ml G418 for 6 days. G418-resistant colonies were picked and screened for homologous recombination by PCR. Targeted embryonic stem cell clones were injected into C57BL/6 blastocysts to generate chimeras. Male chimeras were bred to C57BL6 females to transmit the *Zfyve27-CreERT2* allele. *Neo* cassette was then removed by breeding to ACTB-Flpe deleter mice (Jackson Laboratories; stock



number 019100). Mice colonies were maintained in C57BL6. For genotyping, the following primers were used: forward: GGCCTCATGAAGAGTGATAGGCTTT; reverse: TAATGCAGGCA AATTTGGTGTACG.

Statistical Analysis

Data are shown as mean \pm SEM and were analyzed by Student's *t* test or, when appropriate, ANOVA with Bonferroni correction for multiple comparisons. Where appropriate for transformed values, the Tukey-Kramer multiple comparison test was used (Zar, 1984).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, five tables, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.03.008>.

AUTHOR CONTRIBUTIONS

J.A.O. and R.V.S. designed and performed the experiments and discussed the results. S.J. performed experiments, discussed the results, performed the confocal imaging and organ culture assay, and maintained the mouse lines. Q.-Y.Z., A.D., and W.W. performed experiments and discussed the results. T.H. designed and performed experiments and discussed the results. Q.A. designed experiments and discussed the results. J.A.O. and Q.A. wrote the manuscript.

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