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Palladin is Upregulated in Kidney Disease and Contributes to Epithelial Cell Migration After Injury

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Recovery from acute kidney injury involving tubular epithelial cells requires proliferation and migration of healthy cells to the area of injury. In this study, we show that palladin, a previously characterized cytoskeletal protein, is upregulated in injured tubules and suggest that one of its functions during repair is to facilitate migration of remaining cells to the affected site. In a mouse model of anti-neutrophilic cytoplasmic antibody involving both tubular and glomerular disease, palladin is upregulated in injured tubular cells, crescents and capillary cells with angitis. In human biopsies of kidneys from patients with other kidney diseases, palladin is also upregulated in crescents and injured tubules. In LLC-PK1 cells, a porcine proximal tubule cell line, stress induced by transforming growth factor- β 1 (TGF- β 1) leads to palladin upregulation. Knockdown of palladin in LLC-PK1 does not disrupt cell morphology but does lead to a defect in cell migration. Furthermore, TGF- β 1 induced increase in the 75 kDa palladin isoform occurs in both the nucleus and the cytoplasm. These data suggest that palladin expression is induced in injured cells and contributes to proper migration of cells in proximal tubules, possibly by regulation of gene expression as part of the healing process after acute injury.

Acute kidney injury (AKI) is an abrupt reduction in kidney function with many possible causes, including acute tubular necrosis (ATN). On the cellular level, the pathophysiology of ATN is complex: typically, tubular epithelial cells lose polarity, brush borders are lost, membrane proteins are no longer appropriately localized, the cytoskeleton is disrupted, and the tubular epithelial cells ultimately die and are shed into the urine^{1,2}. Long-term outcomes for patients with ATN are variable, and the factors that determine the ability of an individual patient to recover are not well understood. In fact, there is a lack of agreement about the source of the progenitor cells responsible for repair of tubules^{3,4}. A better understanding of each step in the repair process is necessary for the generation of prognostic biomarkers or therapeutic targets that can ameliorate the devastating effects of AKI from ATN. Our study focuses on gaining insight into the process of kidney injury by studying the function, expression and localization of palladin, a widely-expressed, cytoskeleton-associated protein that has been implicated in the wound-healing process in multiple organs.

Palladin's role in organized tissues has been explored using both a knockout mouse approach and an experimental injury approach. Palladin is necessary for proper embryonic development, as the global knockout mouse has an embryonic lethal phenotype and displays defects in body-wall closure⁵, a process that resembles wound-healing in adults. In injury models, palladin is rapidly upregulated along the wound-edge in the brain, skin and aorta of adult rodents⁶⁻⁸, implicating it in the process of tissue remodeling in these organs; however, palladin's role in kidney disease and injury has not yet been investigated. Previous work has shown that palladin is expressed in multiple cell types in the adult, uninjured mammalian kidney, including smooth muscle cells, mesangial cells and podocytes⁹.

Initial reports describing palladin's expression and sub-cellular localization recognized three distinct palladin isoforms^{10,11}. Additional isoforms have since been identified, and the Universal Protein database now reports the existence of nine variants with predicted molecular masses ranging from 43 to 150 kDa. These isoforms are generated via differential splicing and alternative start-sites¹²; in addition, some cell types generate palladin size-variants by post-translational regulated proteolysis¹³. Previous research has focused largely on the biological role of isoform 4, and to a lesser extent on isoform 3, while the other isoforms have not been studied comprehensively. In our study, we test the hypothesis that palladin isoforms play a role in the kidney's response to acute injury. We

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show that palladin isoform 4 is upregulated in injured or stressed tubular epithelial cells and that palladin is required for appropriate cell migration.

Results

Mouse Kidney Abundantly and Predominantly Expresses Palladin Isoform 4. Palladin was previously detected in the kidney using the monoclonal antibody 1E6, which recognizes epitopes within a proline-rich domain⁹ found only in isoforms 1, 3 and 4 (Figure 1). It is now known that six additional palladin isoforms exist that are not detected by 1E6. To test whether any of the more recently described isoforms of palladin are expressed in the kidney, we utilized two previously characterized pan-palladin polyclonal antibodies (621 and 622)^{14,15}, as well as an antibody (PALL75) targeting a domain contained in isoforms 1, 3 and 4, which provides more consistently reliable results than 1E6. Specificity of PALL75 was tested by immunoblot analysis of previously characterized human pancreatic carcinoma-associated fibroblasts¹⁶. PALL75 detected a robust 75 kDa band, the predicted size of isoform 4, in WT cells and only low levels of this band in the cells in which isoform 4 has been stably knocked down with shRNA (Figure 2A). Of note, in previous work from our lab and others, this band ran at a higher molecular weight, ~90 kDa. This was thought to be due to the presence of proline-rich regions in certain isoforms. With newer gel systems, this isoform is now running closer to its predicted MW of 75 kDa. Immunoblots of lysates of whole mouse kidney with 621 and PALL75 demonstrated that isoform 4 is the major palladin variant in the kidney, although a number of other bands which may be lower prevalence variants were also detected (Figure 2B). These results are consistent with previous studies showing that palladin isoform 4 protein⁹ and mRNA¹⁷ are abundant in the adult mammalian kidney.

Multiple Cell Types Within the Kidney Express Palladin. Immunohistochemical (IHC) staining of adult mouse kidney sections using

the pan-palladin antibody 622 was performed to investigate palladin expression at the cellular level. Low-magnification image (Figure 2C) showed higher levels of palladin expression in the cortex compared to medulla. At higher magnification (Figure 2D), smooth muscle cells and podocytes were palladin-immunopositive, consistent with previously published results⁹. Palladin was also detected in tubular and parietal epithelial cells, which had not been demonstrated previously. IHC with PALL75 revealed that palladin was robustly detected in podocytes and smooth muscle cells (Figure 2, E–G), and also detected in tubular and parietal epithelial cells, confirming the results obtained with the pan-palladin antibody.

Palladin Expression is Altered in Multiple Models of Kidney Injury. To investigate the pattern of palladin expression following injury to the kidney, an *in vivo* mouse model and biopsy specimens from human kidney disease as well as *in vitro* models of injury were utilized. First, palladin expression was investigated using a mouse model of an autoimmune disease that generates chronic injury in the kidney. The α -myeloperoxidase (α -MPO) model of anti-neutrophilic cytoplasmic antibody (ANCA) histologically exhibits both glomerular and tubulointerstitial injury after disease induction by splenocyte transfer¹⁸. Animals were examined at 4, 8, 12, 16 and 19 days after transfer. Two animals were stained from each time point. Four days after transfer, there was no significant glomerular disease activity as assessed by the absence of crescents (Supplemental Figure 1), although tubular injury was already apparent. Twelve days after splenocyte transfer, significant disease activity was observed in the glomerular compartment with active cellular crescents and in the tubular compartment with necrosis, dilation and vacuolization of tubular cells. By day 16, some tubules had healed and crescents were more fibrocellular.

Expression of palladin in injured tubular cells, as assessed by semi-quantitative scoring by a blinded pathologist, increased as severity of injury increased. By day 16, in healed tubules, palladin levels

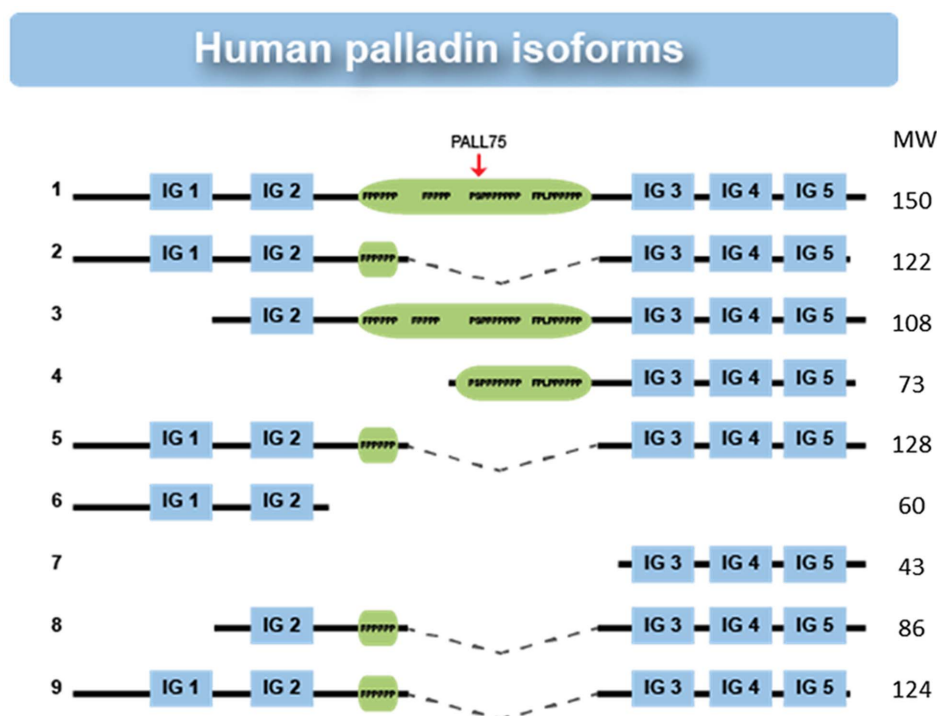


Figure 1 | Nine palladin isoforms. The nine currently identified palladin isoforms and structural domains encompassed by each. Blue boxes denote Ig domains and green boxes denote proline-rich domains with specific proline-rich sequences as indicated. An arrow marks the region used to develop the antibody that recognizes isoforms 1, 3 and 4 using genomic antibody technology (PALL75). Map is adapted from UniProt sequences and utilizes UniProt nomenclature for human palladin isoforms, which exhibits some differences with NCBI, both in nomenclature and sequences of some isoforms. Predicted molecular weights (in kDa) are indicated.

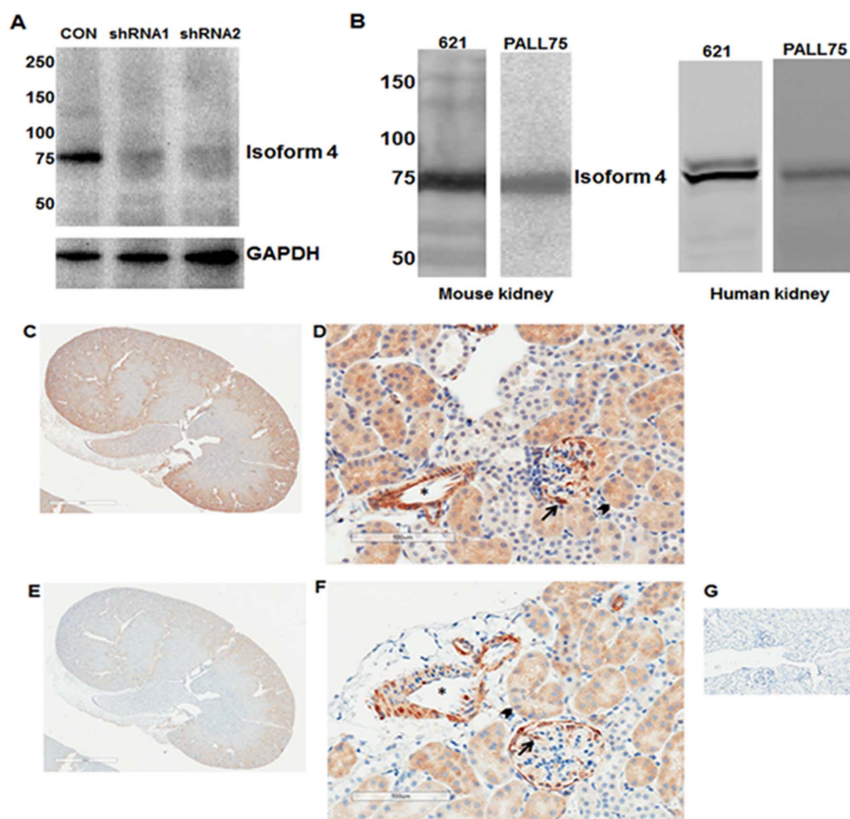


Figure 2 | Validation of PALL75 and palladin isoforms in mouse and human tissue. PALL75 recognizes predominantly a band ~ 75 kDa which is knocked down in stable knockdown lines from human cancer-associated fibroblasts, on which we have previously published and designated shRNA1 and shRNA2¹⁶. A less intense band at ~ 130 kDa is also present, which probably represents isoform 3. Although predicted MW of isoform 3 is ~ 108 kDa, it actually runs at ~ 130 kDa as confirmed by mass spectrometry of excised 130 kDa band in a human cell line (data not shown). GAPDH shown for loading control (A). Different isoforms in mouse and human kidney. Proteins extracted from kidneys of wild type mice and human were immunoblotted with antibodies to palladin. One representative sample (B). 621 detects isoform 4 most robustly. Other bands are present which may represent lower-prevalence variants. PALL75 detects isoform 4 most robustly. Immunohistochemical staining of C57BL/6 wild type mouse kidney with 622 (C, D) and PALL75 (E, F). Vessels are shown with asterisks (*) as palladin is known to be present in smooth muscle cells. Arrows denote podocytes. Arrowheads denote proximal tubule cells. No primary antibody control (G).

returned to near baseline. In the glomerular compartment, cellular crescents present at day 12 expressed high levels of palladin. As crescents became more fibrous than cellular with deposition of extracellular matrix, palladin levels in crescents decreased (Figure 3A). In the vascular compartment, peritubular capillaries, which do not express palladin in wild type mice and 4 days after splenocyte transfer, began expressing palladin in the peritubular capillaries 12 days after injury that partially resolved by day 16 (Figure 3B). Thus, expression of palladin correlated closely with disease activity in this model system in three different compartments. Interestingly, others have previously shown at the mRNA level that palladin is down-regulated in human focal segmental glomerulosclerosis (FSGS)⁹. One possibility for this discrepancy from our findings can be explained by a difference in mRNA expression and protein expression. Additionally, the cell types involved in each type of kidney disease will affect palladin expression levels differently.

The increase in expression of palladin in the mouse model of disease led us to examine its expression and cellular localization in human patients. Biopsy samples exhibiting both tubular and glomerular injury were examined. The arterionephrosclerosis section includes the vascular compartment in which smooth muscle cells and endothelial cells are positive. The tubular and glomerular compartments do not show active disease. The tubular compartment is mostly negative, although distal tubules are faintly positive. The glomerular compartment is positive in podocytes and parietal epithelial cells (PECs) but negative in mesangial cells. The ANCA sec-

tion exhibits crescents and tubular injury. Crescents are intensely positive, and injured tubular cells are also positive. The Lupus section also has crescents, which are intensely positive. The tubular compartment is not injured, and the cells are negative. The anti-glomerular basement membrane (GBM) section also has positively staining crescents and an injured tubular compartment, which is positive. A normal glomerulus is also shown in this section (Figure 3C). As in the mouse model of ANCA, palladin expression in tubules increased in proportion to disease severity, and in three different crescentic diseases, palladin was consistently expressed within crescents. In three ATN patients with non-immunologic kidney disease, expression of palladin in tubular epithelial cells increased with severity of injury (Figure 3D).

Due to the abundance of isoform 4 in the kidney by immunoblot and the increase in palladin expression in the α -MPO mouse model and human kidney diseases, we examined whether isoform-specific upregulation of palladin isoforms may be a common cellular response to cytokines associated with inflammation and injury, regardless of cell type. To address this question, we utilized a commonly used cell-culture model to study the response of proximal tubule cells to injury. Immunoblot analysis revealed that LLC-PK1 cells, derived from the kidney proximal-tubule, express modest levels of isoform 4 at baseline, similar to levels of expression as observed by IHC staining of normal tissue. However, when LLC-PK1 cells were treated with TGF- β 1, isoform 4 levels increased after 2 days of exposure (Figure 4A).

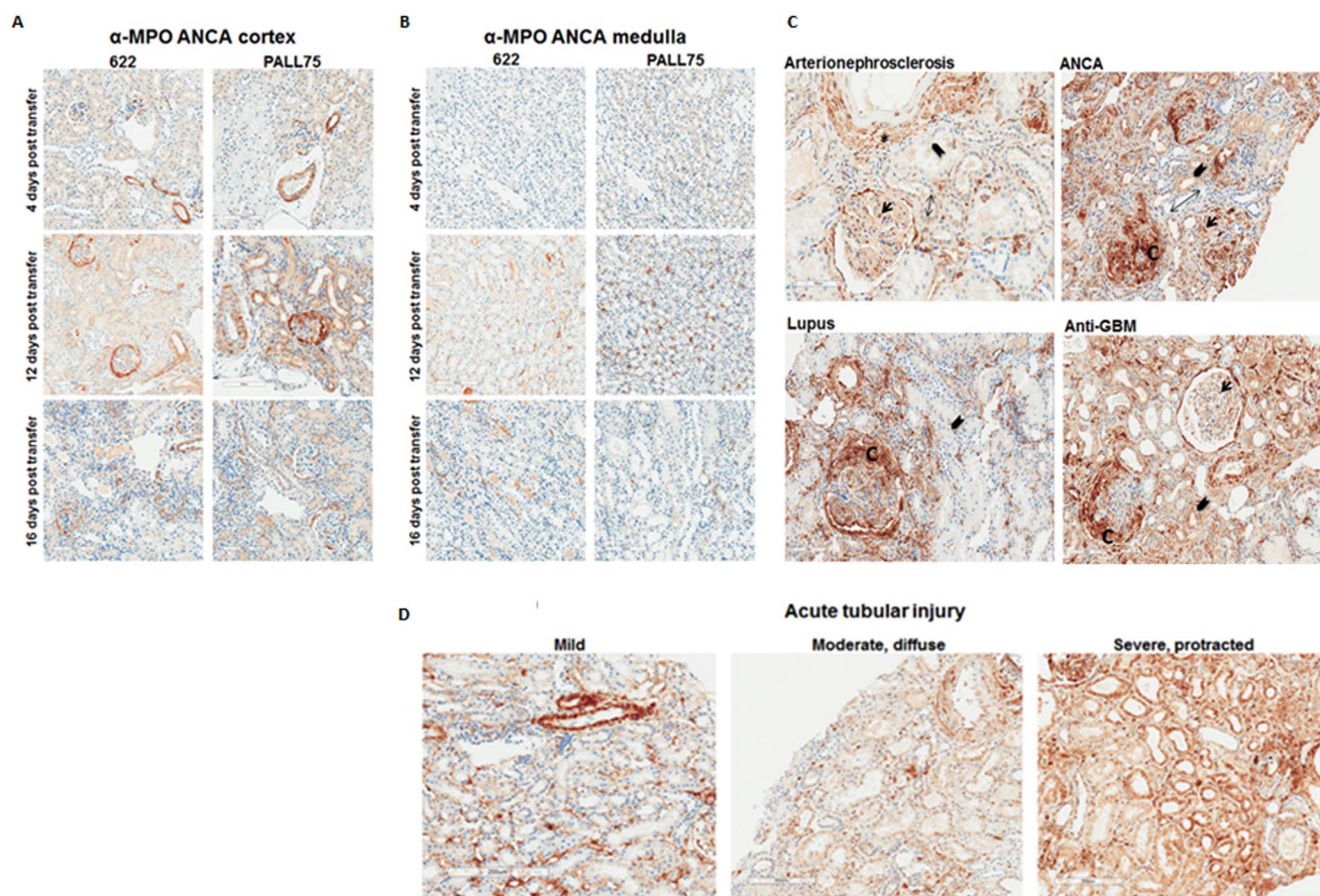


Figure 3 | Palladin expression in α -MPO model of ANCA and human kidney diseases. IHC staining of mouse kidney cortex with 622 and PALL75 (A) show an increase in palladin expression in tubules and crescents as disease progresses from 4 days up to 12 days after disease induction. Sections from medulla show an increase in staining in the vasa recta of the medulla with intensity parallel to disease severity (B). Palladin expression in human biopsy specimens. IHC staining of biopsy sections from different human kidney diseases with PALL75 (C). Arrows denote podocytes; arrowheads denote proximal tubule cells; double-headed arrows denote distal tubule cells; (*) denote smooth muscle cells; C denotes crescents. IHC staining of three patients with non-immunologic mediated kidney injury and ATN with PALL75 (D).

Epithelial-to-mesenchymal transition (EMT) refers to the phenomenon of transformation of a cell from an epithelial to a mesenchymal phenotype. TGF- β 1 is a known mediator of EMT. Previous studies have shown that EMT in LLC-PK1 cells is triggered more effectively by the combined treatment of TGF- β 1 together with disruption of the cells' adherens junctions¹⁹. Reducing cell confluence or lowering the concentration of Ca⁺⁺, combined with TGF- β 1 treatment modeled this “two hit” treatment. Results show a synergistic increase in expression of isoform 4 (Figure 4B).

Because IHC revealed strong expression in glomerular cells, expression of isoform 4 was also examined in an immortalized mouse podocyte line. Isoform 4 was expressed at baseline in podocytes, consistent with IHC results, and treatment of cultured podocytes with TGF- β 1 led to a greater than 50% increase in isoform 4 expression (Figure 4, C and D). Interestingly, a slight increase in expression is also seen in a band ~120 kDa, detected by both 621 and PALL75, which is presumed to be isoform 3.

Palladin Knock Down Does Not Change Morphology of LLC-PK1. Results from mouse and human kidneys showed that palladin is upregulated in response to injury; in pig cells in culture, the primary isoform upregulated is isoform 4, suggesting that isoform 4 could play a role in the morphological remodeling that occurs after injury. To investigate the role of palladin in morphologic remodeling, palladin was transiently knocked down in LLC-PK1 cells using two different siRNAs (4P1 and 4P2) targeted to isoform 4 (Figure 5A). Fluorescence imaging of control and knockdown cells revealed no

obvious morphological differences in the actin cytoskeleton in the palladin knockdown cells (Figure 5, B–D). The ability of knockdown cells to respond to TGF- β 1 with the characteristic increase in abundance and assembly of stress fibers was examined. Surprisingly, knockdown of palladin had no effect on either actin cytoskeleton organization (Figure 5, E–G) or α -smooth muscle actin (α -SMA) protein expression (Figure 5H), a commonly used marker of myofibroblasts, indicating that palladin does not contribute to TGF- β 1-dependent stress fiber formation in LLC-PK1 cells.

Knockdown of Palladin Delays Wound-Healing *In Vitro*. Because palladin has previously been shown to play a role in migration of a fibroblast cell line⁵ and vascular smooth muscle cells²⁰, and because cell migration is required for healing after AKI, we hypothesized that palladin also participates in the migration of tubular epithelial cells. To address this question, we performed a scratch-wound assay in which a wound is introduced into a confluent monolayer of epithelial cells then observed as the remaining cells migrate to refill the artificial wound. Knockdown of palladin in LLC-PK1 cells led to a significant defect in cell migration along the wound edge compared to control (Figure 6 and supplemental videos). Despite our previous results showing that palladin is not required to maintain normal actin organization, these results indicate that it is important in epithelial cell migration *in vitro*, suggesting a distinct molecular function in this cell type.

Palladin Isoform 4 is Present in the Nucleus. An alternative way that palladin may affect cell migration is by regulating gene

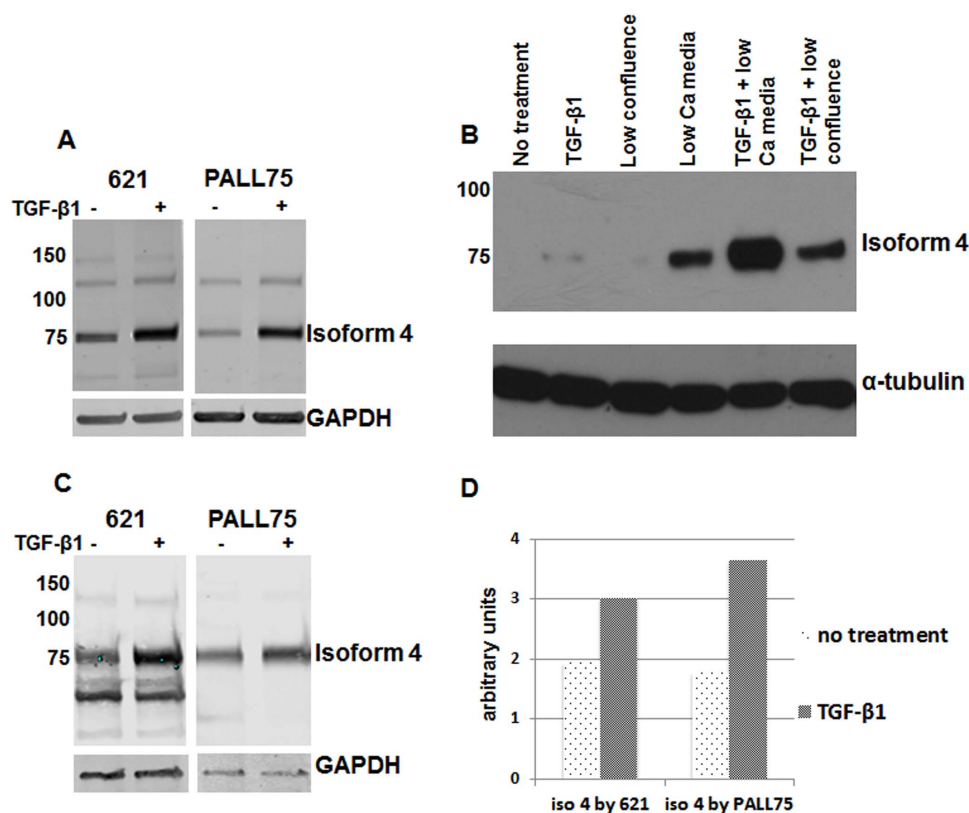


Figure 4 | Increased palladin isoform 4 expression in immortalized proximal tubule epithelial cells, LLC-PK1, and podocytes in response to stress. LLC-PK1 cells were treated for two days with TGF- β 1 and lysed. Cell lysates were run on Western Blot (A). Detection with 621 shows several different isoforms present in the tubular cells with the most significant change in isoform 4. PALL75 also detects an increase in isoform 4 expression (A). GAPDH is shown for loading control. Isoform 4 expression is increased further in a double stress model with TGF- β 1 and low calcium media (B). α -tubulin is shown for loading control. Differentiated immortalized mouse podocytes were treated for two days with TGF- β 1, lysed and run on Western Blot (C). Detection with 621 shows several different isoforms. Isoform 4 is present at baseline and increases by 55% with TGF- β 1 treatment by densitometric analysis (D). When blotted with PALL75, the increase in isoform 4 expression is also seen and confirmed by densitometric analysis (C and D). GAPDH is shown for loading control.

expression. Palladin has been shown to localize to the nucleus in a variety of cell types, and to regulate gene expression in vascular smooth muscle cells^{9,21}. To assess whether palladin could play a similar role in kidney epithelial cells, palladin's subcellular localization was probed by immunoblot analysis of fractionated LLC-PK1 cells. Isoform 4 was detected in both the cytoplasm and the nucleus at baseline, and its levels increased in both compartments following treatment with TGF- β 1 (Figure 7). This novel observation in this cell type is consistent with a model of direct or indirect gene regulation by palladin isoform 4.

Discussion

Palladin is a multi-domain, actin-binding protein that is widely expressed in mammalian organs, including the kidney.^{9,17} It has an unusual molecular niche, as it binds directly to both F-actin and multiple other actin-binding proteins, suggesting that it may function as an actin-binding scaffold protein^{10–12,20,22–27}. In addition to binding actin, it is also a regulator of gene expression^{21,28}, as well as the activity of two different Rho-family GTPases, Rac 1 and Cdc42, both of which play critical roles in actin organization^{16,29}. Palladin also binds directly to multiple proteins that are key regulators of actin dynamics, including profilin²², VASP²³, ezrin¹¹, Lasp-1¹², EPS8²⁹, CLP-36²⁶, and α -actinin^{24,25}. For these reasons, much of the scientific literature to date has focused on palladin's role in organizing actin-based structures, including the invadopodia found in tumor-associated fibroblasts and breast cancer cells^{16,30}; stress fibers, lamellipodia and focal adhesions in mouse embryonic fibroblasts³¹; podosomes

and dorsal ruffles in vascular smooth muscle cells²⁹; and the axonal growth cone in cortical neurons³². In addition, previous work implicated palladin (isoforms 3 and 4 in dermal fibroblasts, and isoform 4 in smooth muscle cells and astrocytes) in the process of wound healing, as specific palladin isoforms are rapidly upregulated at sites of injury to the brain⁸, aorta²⁰, and skin⁷.

Although previous studies have documented the expression of palladin isoform 4 in smooth muscle cells, podocytes and mesangial cells of the kidney⁹, this is the first report documenting palladin expression in other kidney cell types, including endothelial cells, parietal epithelial cells and tubular epithelial cells. Furthermore, our study is the first to examine palladin expression in diseased or injured kidneys. Our results obtained with IHC staining of mouse and human kidney sections, taken together with results obtained using cultured kidney epithelial cells, suggest that palladin plays a role in the healing process after acute kidney injury.

It is well established that LLC-PK1 cells undergo EMT *in vitro* in response to TGF- β 1^{33–35}, a commonly accepted mediator of tubulointerstitial fibrosis. In a previous study of palladin's role in skin injury, dermal fibroblasts were shown to upregulate the expression of palladin isoforms 3 and 4 following treatment with TGF- β 1⁷. Similarly, we observed an increase in isoform 4 expression in LLC-PK1 cells treated with TGF- β 1 with a slight increase in isoform 3. Based on palladin's role in organizing the actin cytoskeleton in other cell types^{5,10,20,32,36}, we expected that knocking down palladin in LLC-PK1 would lead to alterations in actin organization both before and after TGF- β 1 treatment, but surprisingly, we did not observe any

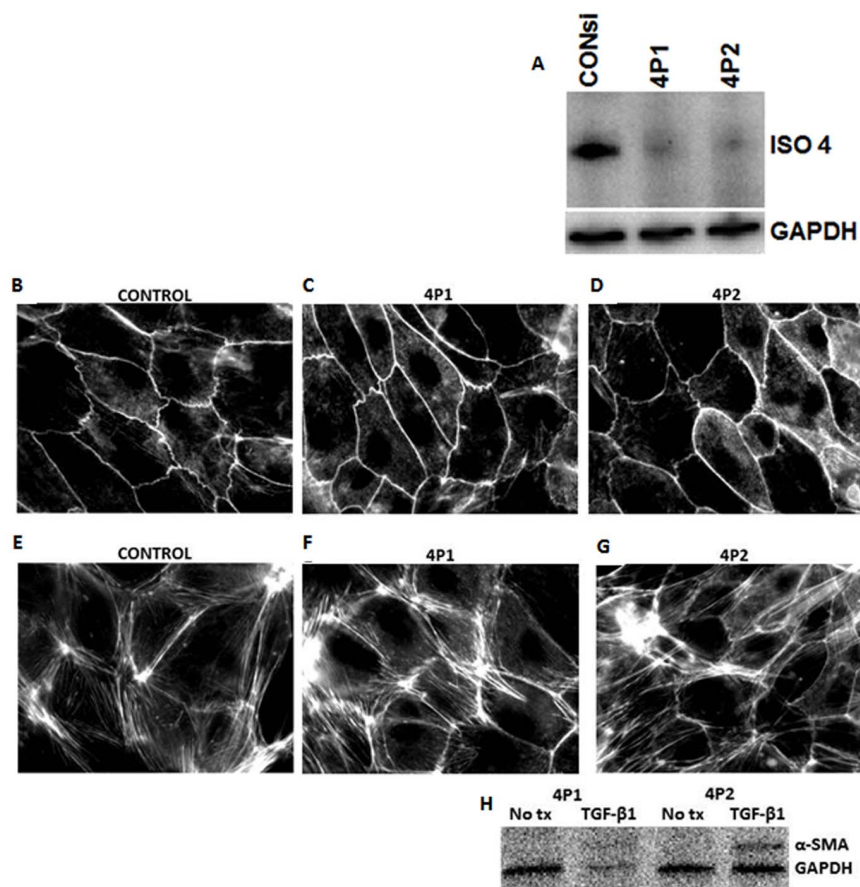


Figure 5 | Palladin knock down does not show change in stress fiber formation in response to TGF- β 1. Western blot confirms efficient transient knockdown of isoform 4 with siRNA (A) in LLC-PK1 cells. GAPDH shown for loading control. LLC-PK1 cells were exposed to either control scrambled RNA sequence (CONsi) or siRNA targeted to palladin isoform 4 (4P1 and 4P2), then fixed and stained with phalloidin. Untreated CONTROL, 4P1 and 4P2 cells (B–D). CONTROL, 4P1 and 4P2 treated with TGF- β 1 (E–G). Western blot shows that the knockdown cells express α -SMA when treated with TGF- β 1 (H).

changes in actin organization in the palladin knockdown cells, suggesting that palladin does not play an essential role in the morphological changes that occur with EMT.

Despite these unexpected results, our *in vivo* findings in the α -MPO mouse model and human kidney biopsies suggest a potential role for palladin in the kidney's response to injury, as expression correlated positively with disease severity. As palladin has been shown to play a role in the migration of smooth muscle cells²⁰, breast cancer cells³⁰, and tumor-associated fibroblasts in culture¹⁶, we hypothesized that isoform 4 could play a critical role in the migration of tubular epithelial cells following injury. Although the origin of these regenerating tubular cells is still under debate^{3,4}, migration is part of the process of healing in the injured kidney^{1,2,37} regardless of the nature of the cells, and this process shares attributes with development, where palladin is known to have a role. Thus, a defect in migration would delay healing from acute tubular injury. Scratch-wound assays demonstrated that palladin was indeed necessary for appropriate migration *in vitro*. Of note, we were unable to specifically target isoform 4 for knockdown as it is completely embedded in isoforms 1 and 3, so we were unable to rule out a contribution from either of these lesser prevalent isoforms. Indeed, isoform 4 is most highly upregulated in our cell culture model, although isoform 3 also shows a slight increase in expression. One interpretation of this finding is that after acute injury, upregulation of palladin isoform(s) is an essential step in the migratory process that allows epithelial cells to move to areas of denuded basement membrane where they regain polarity and re-establish a viable epithelial layer.

There is a growing body of evidence supporting a sub-population of tubular epithelial cells, called scattered tubular cells, that have the capacity to regenerate in response to acute kidney injury. Astonishingly, these cells share similar markers (CD133 and CD24) and protein expression patterns with PECs^{3,38}. The expression pattern of palladin in tubular epithelial cells after injury and PECs suggests that it may be another marker of this cell type, although its presence in other cell types limits its specificity. Both cell types migrate, either to the site of injury as with scattered tubular cells, or to the glomerular crescent, as is proposed in crescentic glomerular diseases.

If indeed palladin plays a role in migration and in differentiation of smooth muscle cells²¹ as others have shown, this leads to the question of whether palladin is more than just a scaffolding protein. Palladin contains no nuclear import signals, but it does contain a nuclear export signal and has been shown in both podocytes and differentiated smooth muscle cells to accumulate in the nucleus when nuclear export is blocked with leptomycin B²¹. In addition, recent studies showed specific point mutations at sites of actin binding, which led to nuclear localization³⁹. A co-immunoprecipitation assay showed binding to myocardin-related transcription factors (MRTFs), and imaging colocalized palladin to MRTFs²¹ in differentiated smooth muscle cells. Furthermore, knock down of palladin in these cells altered expression of proteins contributing to their contractile properties³⁶. Our results showed that an increase in nuclear expression of isoform 4 also occurs in a stress model *in vitro*. Although further investigation is required, this finding suggests palladin may play a role in gene regulation.

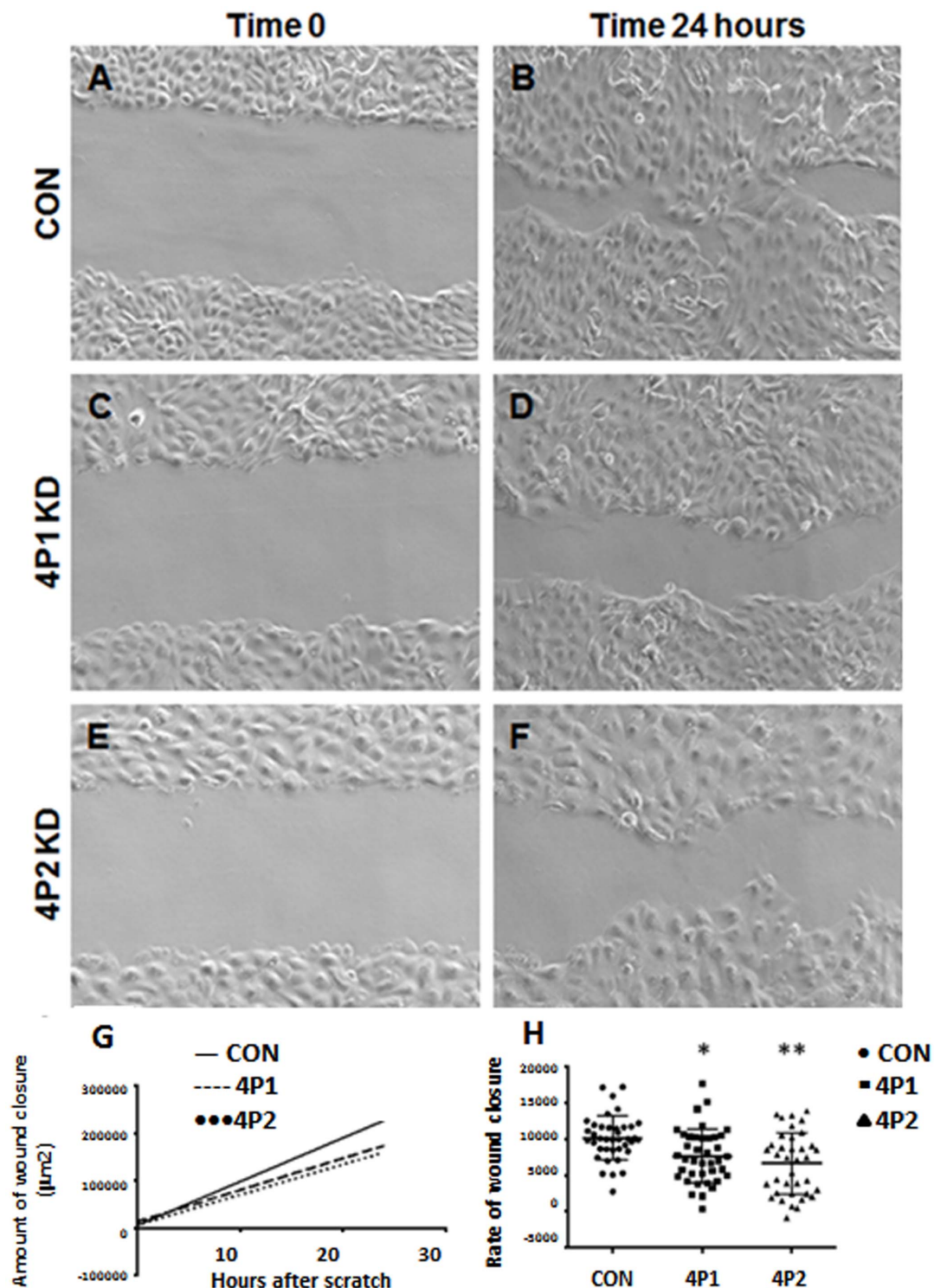


Figure 6 | Scratch wound assay shows defect in migration in palladin deficient cells. LLC-PK1 cells transfected with control scrambled sequence, 4P1 or 4P2 were subjected to scratch-wound assay. Initial and final images are shown for control sequence (A and B), 4P1 (C and D) and 4P2 (E and F). Data from five separate scratch assays were aggregated from both the palladin knock down cells and the cells transfected with control scrambled sequence. Linear regression lines represent the amount of closure determined from each time point (G). The slope represents the rate of closure of each individual scratch point for CON, 4P1 and 4P2 (H). Each dot represents one scratch point analyzed. * $P=0.0019$ for CON vs. 4P1; and ** $P=0.0001$ for CON vs. 4P2.

One limitation of this study is the correlative nature of our findings. Disease-induced increases in palladin expression could be either protective or harmful. Findings by scratch assay suggest a protective role, but this hypothesis cannot be confirmed without an *in vivo* knockout, which poses a unique challenge as the global knockout of all palladin isoforms is embryonic lethal and cannot be

used for functional studies. The presence of multiple isoforms with overlapping regions also makes developing certain isoform-specific knockouts a challenging endeavor. In addition, expression in multiple cell types necessitates multiple cell-specific knockouts. However, determining palladin's function in the injured kidney will shed insight into its role as a potential therapeutic target.

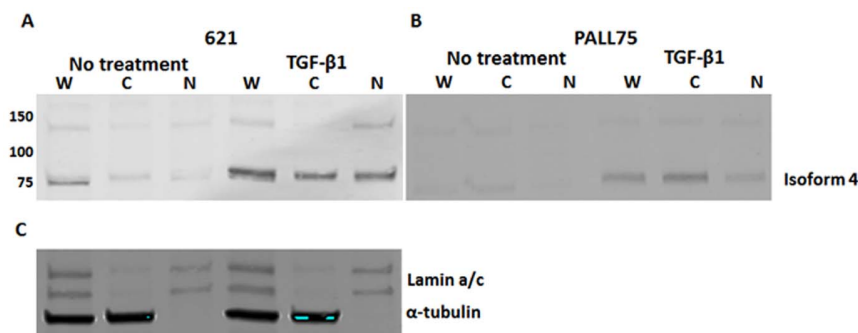


Figure 7 | Nuclear fractionation of palladin isoform 4. Whole cell and fractionated lysates of LLC-PK1 cells treated with TGF- β 1. Blot with 621 (A) and PALL75 (B) shows an increase in isoform 4 expression in whole cell lysates (W) and in both cytoplasmic (C) and nuclear (N) compartments. Lamin and α -tubulin blots confirm fractionation (C).

Additionally, regardless of whether it contributes to or prevents injury, if the level of palladin expression in tubular epithelial cells correlates with disease severity as suggested by IHC, it also has potential as a biomarker. Future studies to assess whether palladin increases in tubular cells shed into the urine during episodes of AKI could provide a useful, non-invasive tool for diagnosis and prognosis of AKI.

In summary, our study is the first to show an increase in palladin expression in the kidney in different cell types of each compartment (tubular, glomerular and vascular) and in different diseases, suggesting a role for palladin in pathogenesis of kidney disease of various etiologies. In addition, our *in vitro* studies suggest that palladin may have a protective role in the tubular compartment as it is necessary for proper migration. Further understanding of the precise role of palladin in kidney disease will help advance knowledge of the pathogenesis of AKI and reveal a role for palladin as a therapeutic target or biomarker.

Methods

Cell Lines. The kidney proximal tubule cell line, LLC-PK1 from ATCC (ATCC# CL-101) has been previously characterized as having properties of kidney proximal tubule cells^{40,41}. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For serum starvation, cells were grown in DMEM supplemented with 0.5% FBS and penicillin/streptomycin 12–18 hours prior to the experimental protocol. All cell lines were maintained at 37°C with constant 5% carbon dioxide.

The heat sensitive mouse podocyte line was a kind gift from Dr. Stuart Shankland and maintained in RPMI containing 10% FBS and sodium pyruvate (Gibco, Grand Island, NY) and maintained as described⁴². Cells were held in proliferation stage at 33°C with interferon- γ (Roche, Indianapolis, IN) and temperature switched to 37°C to induce differentiation 10–12 days prior to cell lysis.

Animal Model. The mouse model of antineutrophilic cytoplasmic autoantibody (ANCA) specific for myeloperoxidase (MPO) was previously published¹⁸ and is referred to here as α -MPO. In brief, α -MPO knockout mice received MPO injection, with initial priming intraperitoneal injection of 10 μ g followed by boosters at days 21 and 36. They were given a final 10 μ g intravenous injection four days prior to transfer of 1×10^5 α -MPO splenocytes from these animals to recombinase-activating gene-2 (Rag2) deficient mice which lack functioning B and T lymphocytes. Animals were sacrificed at 4, 8, 12, 16 and 19 days after splenocyte transfer. Severe necrotizing and crescentic glomerulonephritis developed. Kidneys were fixed in 10% formalin and sectioned for staining. Animal studies were performed under protocols in accordance with relevant guidelines and regulations and approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

Antibodies. The following antibodies were used: palladin monoclonal 1E6 (Novus Biologicals, Littleton, CO, USA); GAPDH (GeneTex, San Antonio, TX, USA and Santa Cruz Biotechnology, Dallas, TX, USA); α -smooth muscle actin (Abcam, Cambridge, MA, USA); α -tubulin (Thermo-Scientific, Rockford, IL, USA); α -lamin A/C (Sigma Aldrich, St. Louis, MO, USA) and palladin polyclonals 621 and 622. These antibodies were generated by immunizing rabbits with multiple fusion proteins representing N-terminal, central and C-terminal fragments of palladin isoform 1. 621 performed optimally on western blots, and 622 performed optimally in immunohistochemical staining, and were used accordingly. HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove,

PA, USA). IR dye secondary antibodies were obtained from LICOR (Lincoln, NE, USA).

A polyclonal antibody directed to specific target sequences from full-length human palladin was generated using genomic antibody technology by SDIX (Newark, DE, USA). DNA sequence from the target region of amino acids 713–802, including one of the proline-rich regions, was incorporated into DNA to allow for native folding of the epitope of interest and is referred to here as PALL75 (Figure 1).

siRNA Knockdown. The following siRNA were used in this study (sense strands): 4P1 (5' AGGUGAAGCUGAAGCAUUAUU 3') and 4P2 (5' CCGAAGAUUCACUGGUUUUAUU 3'). Their target sequences are contained in isoforms 1, 3 and 4. siCONTROL Non-Targeting siRNA #2 was obtained from Dharmacon (Lafayette, CO). Cells were transfected using the TransIT siQuest transfection reagent (Mirus, Madison, WI) following manufacturer's instructions. Briefly, 6 μ l of transfection reagent was added to 250 μ l serum-free media per 1.5 ml of final volume of media. To this mixture, the appropriate volume of siRNA was added to achieve a final concentration of 40 nM and incubated for 15 minutes at room temperature then added drop-wise to cells. Cells were assayed between 48 to 72 hours post transfection.

Cell Culture. Cells were seeded in 6 well plates and serum-starved overnight prior to treatments. Cells were either treated with TGF- β 1 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 4 ng/ml or carrier. After the designated time period, cells were lysed for immunoblot, and cover slips were fixed for immunofixation.

Scratch-Wound Assay. LLC-PK1 cells were exposed to siRNA KD or control scrambled sequence for 48–96 hours and cultured to confluence in 6 well plates. When confluent, scratches were made with a sterile 200 μ l pipette tip, intersecting at the center of the well. Each well was washed 3x with 1x PBS. Media was replaced on the cells and the plate was placed on an Olympus IX70 microscope in an incubator maintained at 37°C with constant CO₂. Cells were imaged at 10x every 5 minutes for 24 hours using Velocity software and a Hamamatsu ORCA C4742-95 camera. The rate of scratch closure was determined using Tscratch software developed by the Koumoutsakos group (CSE Lab, ETH Zürich⁴³).

Student's t-tests were used for comparing the means of the rate of closure of scratch wounds from five different experiments between cells transfected with control-scrambled sequence and the two transiently knocked down cells lines, 4P1 and 4P2.

Cell and Tissue Lysis and Immunoblot. Cells cultured on 6 well, 60 mm or 100 mm tissue culture dishes were washed with 1x PBS then scraped into a lysis buffer containing 1x PBS, 1% Triton X-100, and protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Supernatant was collected after centrifugation at 14 500 rpm for 15 min at 4°C and analyzed by immunoblot. For the immunoblot, lysates were boiled in 5X Laemmli buffer for 4 minutes, and 20–30 μ g of protein were resolved by SDS-PAGE in each lane of a 4–15% gel then transferred to nitrocellulose and immunoblotted. Immunocomplexes were visualized using the Immobilon Chemiluminescence HRP Detection kit (Millipore, Billerica, MA, USA) or the Odyssey Imaging System for LICOR secondary antibodies (Lincoln, NE, USA).

Kidney tissue was snap-frozen in liquid nitrogen, ground with a chilled mortar and pestle, and extracted in lysis buffer containing 50 mM Tris pH 7.5, 8 M urea, 5% SDS, 10 mM EDTA, and protease inhibitor cocktail. Samples were then centrifuged at 14 500 rpm for 15 min at 4°C to remove any insolubilized particulates, and the supernatant was resolved by SDS-PAGE in a 4–15% gel, using 20 μ g protein per lane. Proteins were transferred to nitrocellulose and immunoblotted and visualized as above.

Immunofluorescence. Cells were grown on glass coverslips and fixed in 4% paraformaldehyde in PBS for 7 minutes, then permeabilized in 0.2% Triton X-100 for 2 minutes, blocked with normal goat serum for one hour and incubated with phalloidin to stain actin. Coverslips were examined with a Nikon TE2000 microscope



with 20x and 60x objective lenses, and a Hamamatsu Orca-ER camera. Images were acquired and analyzed with Metamorph software.

Immunohistochemistry. De-identified patient samples of kidney biopsy cores were obtained through an Institutional Review Board (IRB)-exempt study supported through the Nephropathology Department. The study was reviewed by the University of North Carolina IRB and determined to be exempt from continuing review. Experiments were conducted in accordance with relevant guidelines and regulations. Mouse kidney was prepared as above. Ten total mice were stained, two at each time point of 4, 8, 12, 16 and 19 days post splenocyte transfer. IHC was carried out in the Bond fully-automated slide staining system (Leica Microsystems Inc. Norwell, MA, USA). Slides were deparaffinized in Bond Dewax solution (AR9222) and hydrated in Bond Wash solution (AR9590). Antigen retrieval was performed for 30 min at 100°C in Bond-epitope retrieval solution 1 pH 6.0 (AR9961). After pretreatment, the primary antibodies 622 and PALL75 were applied for 0.5 and 1 hour, respectively. Detection of PALL75 was performed using the Bond Intense R Detection System (DS9263) supplemented with the goat anti-Rabbit-HRP (EnVision+, DAKO, Carpinteria, CA, USA) and Bond Polymer Refine Detection System (DS9800) for 622. Stained slides were dehydrated and cover-slipped. Positive and negative controls (no primary antibody) were included for each antibody. Stained slides were digitally imaged at 20× magnification using the Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA). Digital images were stored in the Aperio Spectrum Database.

Intensity of palladin staining was scored by a blinded pathologist on a semi-quantitative scale (neg, trace, 1+, 2+, 3+). Severity of tubular injury was also assessed by a blinded pathologist on a semi-quantitative scale (normal, mild, moderate and severe) based on the following criteria: loss of brush border, simplification, dilation, irregularity of tubules and denudation of the epithelial cells.

Nuclear/Cytoplasmic Fractionation. Cells were fractionated by first incubating in hypotonic buffer (1 M Hepes, pH 7.9; 1 M MgCl₂; 1 M KCl; 100 mM DTT; 100 mM Na₂VO₄; 0.5 M EDTA with protease inhibitors) on ice for 20 minutes followed by centrifugation at high speed at 4°C to extract the cytoplasmic fraction. The pellet was washed once with hypotonic buffer and once with PBS. This was followed by a 20-minute incubation on ice with a high salt buffer (1 M Hepes, pH 7.9; 1 M NaCl; 1 M MgCl₂; 100 mM DTT; 100 mM Na₂VO₄; 0.5 M EDTA, 50% glycerol with protease inhibitors) and centrifugation at high speed at 4°C to extract the nuclear fraction. Fractionation was confirmed with immunoblot expression of α -tubulin for cytoplasmic and α -lamin A/C for nuclear fraction.

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Author contributions

E.C. wrote the main manuscript. A.G. reviewed all the pathology images. M.K. and J.P. provided technical advice and support. S.G. performed preliminary experiments. R.F. provided general scientific guidance. C.J. provided human and mouse tissue. C.O. supervised overall direction of project. All authors reviewed the manuscript.

Additional information

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