

p21-Activated Kinase 4 and Ischemic Acute Kidney Injury in Mice and Humans

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Key Points

- p21-activated kinase 4 (PAK4) phosphorylates and inactivates GSH peroxidase 3 in the kidney after ischemia-reperfusion.
- Mice lacking PAK4 or treated with PAK4 proteolysis-targeting chimera were protected from kidney damage caused by ischemia-reperfusion injury.
- PAK4 expression in kidney tissues post-transplant inversely correlated with kidney function.

Abstract

Background AKI after ischemia-reperfusion remains a substantial perioperative challenge lacking effective treatment. p21-activated kinase 4 (PAK4), a downstream effector of Rho GTPase, has been explored in hepatic ischemia-reperfusion injury, but its role in renal ischemia-reperfusion is unknown.

Methods Wild-type and proximal tubule-specific *Pak4* knockout mice underwent 25 minutes of ischemia followed by 24 hours of reperfusion injury. Primary tubular cells and human kidney-2 cells were exposed to hypoxia-reoxygenation injury to investigate the *in vitro* effect of PAK4. Selective degradation of PAK4 was employed using proteolysis-targeting chimera (PROTAC) to ameliorate AKI.

Results Post-ischemia-reperfusion, the expression of PAK4 was upregulated through hypoxia-inducible factor 1 α in mouse kidneys. Deletion of PAK4 in proximal tubule cells, but not in myeloid cells, significantly mitigated ischemia-reperfusion-induced AKI, as evidenced by decreased levels of BUN, creatinine, tubular necrosis, apoptosis, macrophage infiltration, and lipid accumulation compared with control mice. Further investigation revealed that PAK4 phosphorylated GSH peroxidase 3 (GPx3) at T47, leading to its proteasomal degradation. In addition, pretreatment of mice with the PAK4 PROTAC preserved GPx3 and enhanced fatty acid β -oxidation, thereby protecting against AKI. In kidney tissues from people with a kidney transplant, elevated levels of PAK4 protein and phosphorylation of GPx3 at T47 were observed.

Conclusions Renal tubular PAK4 contributes to tissue damage during ischemia-reperfusion injury, whereas PAK4 PROTAC mitigates ischemia-reperfusion injury by reducing oxidative stress and promoting fatty acid β -oxidation.

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Introduction

Renal ischemia-reperfusion (I/R) injury stands as a predominant contributor to the occurrence of AKI.^{1,2} Patients facing cardiovascular shock, decompensation of congestive

heart failure, cardiac bypass surgery, or kidney transplantation confront an approximate 50%–80% risk of developing ischemic AKI.³ Severe renal I/R injury after kidney transplantation can result in acute or chronic rejection and,

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in some cases, graft failure by triggering inflammation and oxidative stress.⁴ Over the past few decades, the rates of I/R injury-related AKI have steadily increased worldwide, imposing a growing health care burden. Unfortunately, there is currently no targeted therapy for I/R-related AKI beyond hemodynamic optimization.

Renal tubular epithelial cells have a pivotal role in reabsorbing vital substances filtered from the bloodstream and excreting waste into urine. This process demands a robust energy supply and relies heavily on mitochondrial fatty acid β -oxidation.⁵ In cases of I/R-induced AKI, fatty acid β -oxidation is hindered, leading to an energy shortage and an accumulation of intracellular triglycerides, which in turn contributes to mitochondrial damage and apoptotic cell death.^{6,7} As a result, renal tubular epithelial cells are the most susceptible intrinsic cells to hypoxia, waste product, or mitochondrial dysfunction. Moreover, mitochondrial fatty acid β -oxidation is closely associated with maintaining redox balance.⁸ Several studies have indicated that the disruption of fatty acid β -oxidation in renal tubular epithelial cells results in an excessive buildup of reactive oxygen species (ROS), exacerbating renal tubular cell death, inflammation, and ultimately kidney dysfunction.^{9–11} Therefore, focusing efforts on regulating fatty acid energy metabolism represents an effective strategy for reducing ROS production and preventing the development of AKI.

Crucial antioxidant enzymes known to defend renal tubular cells against oxidative stress include SOD, catalase, and GSH peroxidase (GPx).¹² GPx plays a role in breaking down hydrogen peroxide into water and oxygen by using GSH as a reducing agent.¹³ Interestingly, in several *in vitro* studies, GPx alone has been demonstrated to offer more substantial protection against oxidative stress compared with SOD, catalase, or the combination of SOD and catalase.¹⁴ Thus far, humans have eight different isoforms of GPx, with GPx1 being the most abundant version found in the cytoplasm of nearly all mammalian tissues, and GSH peroxidase 3 (GPx3) being abundant in plasma.¹⁵

The p21-activated kinases (PAKs) are part of a group of serine/threonine kinases initially identified as downstream effectors of Rho GTPases Cdc42 and Rac1.¹⁶ PAKs are categorized into two groups: group 1 PAKs (PAK1–PAK3) and group 2 PAKs (p21-activated kinase 4 [PAK4]–PAK6) on the basis of their sequences and functions.¹⁷ The overexpression of PAKs is commonly found in various types of cancer tissues and is often linked to increased invasiveness and a poor prognosis.¹⁸ One distinguishing feature among different PAK family members is their expression patterns. Among these, PAK4 is expressed in all tissues and is notably upregulated under hypoxic conditions.¹⁸ Recent research has shown a positive association between hypoxia-inducible factor (HIF-1 α) and the translation of PAK4, which has significant implications in cancer because hypoxic regions are common in rapidly growing solid tumors.¹⁹ Similarly, we have reported HIF-1 α -induced PAK4 expression in liver tissues affected by I/R injury.²⁰ Specifically, we noted a substantial increase in PAK4 expression at both mRNA and protein levels in liver tissues affected by I/R injury in mice and in humans with a liver transplant. However, the functional role of PAK4 in kidney tissues during I/R-related AKI remains unclear. In light of

this, our study aimed to elucidate the potential involvement of PAK4 in renal pathophysiology under hypoxic conditions using three different approaches. First, we generated mice with specific PAK4 deficiency in renal proximal tubules and subjected them to renal I/R injury. Second, we developed an orally bioavailable PAK4 proteolysis-targeting chimera (PROTAC) to selectively degrade PAK4 protein in mice. Finally, we analyzed human tissues from kidney transplant patients to examine the correlation between PAK4 protein levels and transplantation outcomes.

Methods

Animals

Pak4^{flox/flox} mice and *Ggt1-Cre* mice were crossed to generate proximal tubule-specific *Pak4* knockout (KO) mice (*Pak4*^{flox/flox}; *Ggt1-Cre*). AKI was induced either by 25 minutes of ischemia followed by 24 hours of reperfusion injury or through intraperitoneal injection of cisplatin (0.5 mg/kg). The study protocol was approved by the Institutional Animal Care and Use Committee of Jeonbuk National University Hospital (Permit No: JBUH-2021-14).

Patients

Human kidney tissue was procured during kidney transplantations. Among the 15 human kidney tissues collected, eight were from living donors and seven were from deceased donors. Punch needle core biopsies were collected from all transplanted kidneys before and after reperfusion. All patients granted written informed consent, and the study received approval from the Institutional Review Board of Jeonbuk National University Hospital (Permit No: JBUH-2022-02-029).

Statistics

Data are expressed as the mean $\pm$ SD. The primary outcomes of animal studies (BUN, creatinine, and histologic parameters) were statistically powered to achieve an α error <5% and power of >80%. All cohorts had more than the minimum required number of mice per group, and our sample sizes are similar to those reported in previous publications.^{21,22} For comparisons between two groups, the Student unpaired *t* test was used. Comparisons among multiple groups were performed using one-way ANOVA with Bonferroni *post hoc* analysis. The Pearson correlation coefficients were calculated for continuous variables. A *P* value < 0.05 was considered significant.

Additional Methods

Detailed methods are provided in [Supplemental Methods](#) and [Supplemental Tables 3–5](#).

Results

PAK4 Expression Was Increased in Renal Tubules after I/R Injury in Mice

To investigate the potential involvement of PAK4 in kidney I/R injury, we initially compared PAK4 expression levels in kidney tissues from control and I/R-injured mice. The

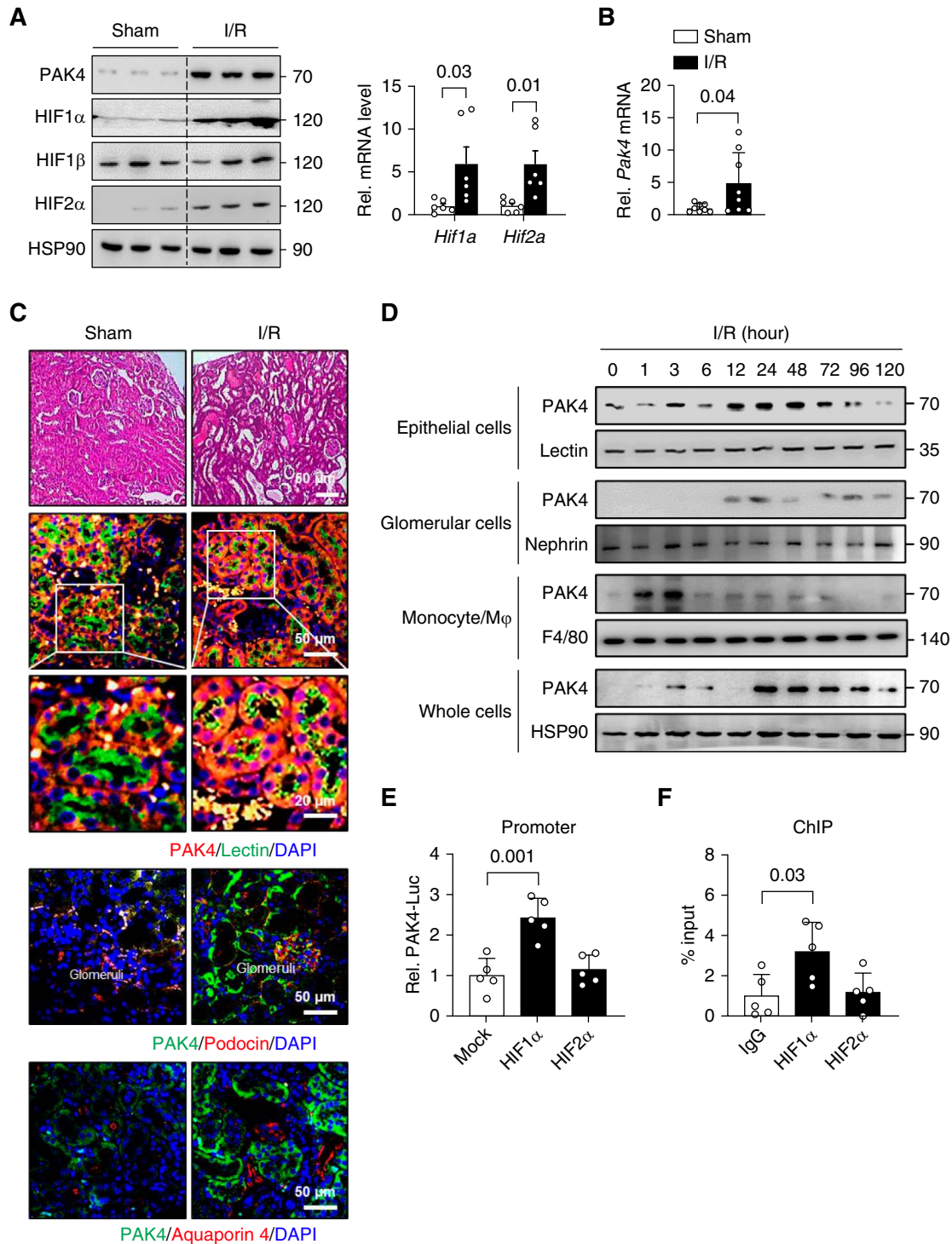


Figure 1. PAK4 upregulation during renal I/R injury in mice. (A and B) Protein and mRNA levels of PAK4 and HIF in WT kidneys after renal I/R ($n=6$ or 8). (C) Immunofluorescence staining of kidney sections for PAK4, *Lotus tetragonolobus* lectin (lectin), podocin, and aquaporin 4. (D) Analysis of PAK4 protein levels over time in kidney tissue cells after I/R. (E) PAK4-luciferase activities were determined in HK-2 cells transfected with mock, HIF-1 α , or HIF-2 α overexpressing construct ($n=5$). (F) ChIP-qPCR assay showing binding of HIF-1 α or HIF-2 α to the *Pak4* promoter ($n=5$). Values are mean $\pm$ SD. ChIP-qPCR, chromatin immunoprecipitation–quantitative polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole; HIF, hypoxia-inducible factor; HK-2, human kidney-2; I/R, ischemia-reperfusion; PAK4, p21-activated kinase 4; WT, wild-type.

results revealed increased protein and mRNA levels of PAK4 in response to renal I/R insult (Figure 1, A and B). On histologic analysis, proximal tubule-specific PAK4

upregulation, identified in *Lotus tetragonolobus* lectin-positive tubules with PAK4 expression, was increased in the kidneys of mice subjected to I/R (Figure 1C). The

increase in PAK4 expression in tubular cells was evident as early as 12 hours post-I/R and persisted up to 72 hours (Figure 1D).

On the basis of our prior report suggesting the regulation of PAK4 expression by HIF-1 α in mouse hepatocytes after I/R injury,²⁰ we subsequently investigated the involvement of HIF-1 α in the regulation of PAK4 transcription within I/R-injured kidney tissues. The significant induction of both HIF-1 α and HIF-2 α by I/R was confirmed (Figure 1A). Overexpression of HIF-1 α , in contrast to HIF-2 α , enhanced the transcriptional activity of PAK4 in luciferase assays and its binding to the promoter of the *Pak4* gene in chromatin immunoprecipitation assays (Figure 1, E and F).

Proximal Tubule-Specific Deficiency of PAK4 Mitigated I/R-Induced and Cisplatin-Induced AKI

The role of PAK4 in the progression of I/R injury was further investigated using mice with proximal tubule-specific *Pak4* KO mice (Figure 2A and Supplemental Figure 1, A and B). Both *Pak4* KO mice and their wild-type (WT) littermates were subjected to renal I/R injury (Supplemental Figure 1C). Notably, *Pak4* KO mice exhibited significantly decreased BUN and creatinine levels compared with the control group (Figure 2B). Histologic analysis revealed that I/R insult led to proximal tubule loss, necrosis, protein casts, apoptosis, and interstitial inflammation, and these effects were significantly attenuated in *Pak4* KO mice (Figure 2C). Western blotting analysis of kidney tissues supported histologic findings (Figure 2D and Supplemental Figure 1D). ELISA of plasma and qPCR of kidney tissues showed increased inflammatory cytokines after I/R, which were reduced in *Pak4* KO mice (Supplemental Figure 1, E and F). To confirm these findings, a second AKI model was used. After cisplatin injection, *Pak4* KO mice demonstrated relatively preserved biochemical, histologic, and Western blot findings compared with WT mice (Supplemental Figure 2). By contrast, mice lacking PAK4 in myeloid cells (*Pak4^{lox/lox}; LysM-Cre*) exhibited a similar degree of tubular injury as littermate control mice subjected to renal I/R (Supplemental Figure 3).

PAK4 Deficiency Enhanced Antioxidant Capacities

To corroborate the involvement of antioxidant capacities in the protective effects of PAK4 deficiency against renal I/R injury, we assessed serologic markers of oxidative stress and levels of antioxidant enzymes in the kidneys. In comparison with WT mice, *Pak4* KO mice exhibited significantly lower levels of malondialdehyde and GSH disulfide, along with significantly higher levels of reduced GSH (Figure 3, A and B). Among the examined antioxidant proteins, SOD 1, SOD 3, and GPx3 exhibited a significant upregulation in kidney tissues of I/R-injured *Pak4* KO mice (Figure 3C and Supplemental Figure 4). Consistently, GPx enzyme activity in kidney tissues and blood was significantly increased in *Pak4* KO mice (Figure 3D). Similarly, in response to hypoxia-reoxygenation (H/R) challenge, there were increases in enzyme activity of GPx and protein level of GPx3 in PAK4-silenced HK-2 cells (Figure 3, E and F).

To identify other potential PAK4 substrates, we conducted a phosphoproteomic analysis using kidney tissues from I/R-injured *Pak4* KO and WT littermates (Supplemental Figure 5A). Employing criteria (a >2-fold change in phosphorylation state with $P < 0.05$), the top-ranked candidates included calreticulin, spectrin, HSP60, and GPx3 (Supplemental Figure 5B). Calreticulin, spectrin, and HSP60 were not studied further because coimmunoprecipitation showed only a slight increase in their levels with PAK4 during H/R conditions (Figure 4A). Remarkably, GPx3 levels significantly increased in PAK4 immunoprecipitates under the same conditions. Intriguingly, when PAK4 was silenced, there was no discernible effect on GPx3 transcription. This was evidenced by the levels of *Gpx3* mRNA, the absence of any effect on GPx3 luciferase activity, and the binding of PPAR γ or SP1 to the *Gpx3* promoter (Supplemental Figure 6, A–C). Similarly, examination of the human Genotype-Tissue Expression (GTEx) database revealed no correlation between *PAK4* and *GPX3* mRNA levels in the kidney tissues of 85 individuals (Supplemental Figure 6D). Consequently, our investigation shifted toward scrutinizing PAK4's regulation of GPx3 protein.

PAK4 Phosphorylated and Destabilized GPx3

Both PLA and *in vitro* kinase assay demonstrated the effective binding and phosphorylation of GPx3 by PAK4 (Figure 4B and Supplemental Figure 7A). The heightened interaction between PAK4 and GPx3, as opposed to GPx4, was additionally validated in I/R-injured kidney tissues (Supplemental Figure 7B). Liquid chromatography–mass spectrometry/mass spectrometry analysis identified T47 as a phosphorylation site on GPx3 by PAK4 (Supplemental Figure 7C and Supplemental Table 1). A peptide competition *in vitro* kinase assay confirmed that the peptide comprising the T47 region (P1) efficiently inhibited GPx3 phosphorylation by PAK4, whereas the nonspecific negative peptide (P') or peptide comprising T165 (P2) had little to no inhibitory effect (Figure 4C). Using X-ray crystallography structures of PAK4 and GPx3 proteins, we predicted a protein–protein docking model for the phosphorylation of GPx3 at T47 (GPx3-T47) by PAK4, confirming its structural feasibility (Figure 4D).

To ascertain the direct causality of T47 phosphorylation in GPx3 protein levels, we generated phospho-deficient and phospho-mimetic mutants by substituting threonine to alanine (GPx3^{T47A}) or aspartate (GPx3^{T47D}), respectively, at the T47 residue. Remarkably, in a cycloheximide chase experiment, the GPx3^{T47A} mutant exhibited increased protein stability compared with GPx3^{WT} and GPx3^{T47D} in response to H/R (Figure 5A). Consistently, PAK4 overexpression with GPx3^{WT}, not with GPx3^{T47A}, markedly increased GPx3 ubiquitination in the presence of proteasome inhibitor MG132 (Figure 5B). To identify potential E3 ubiquitin ligases responsible for GPx3 degradation, we used bioinformatics approaches using Ub browser 3.0 (http://ubibrowser.bio-it.cn/ubibrowser_v3/). Out of the potential candidates investigated, the transfection of small interfering RNA targeting Parkin RBR E3 ubiquitin protein ligase (PRKN) demonstrated a nearly complete inhibition of GPx3 ubiquitination and subsequent degradation triggered by H/R (Supplemental Figure 8A),

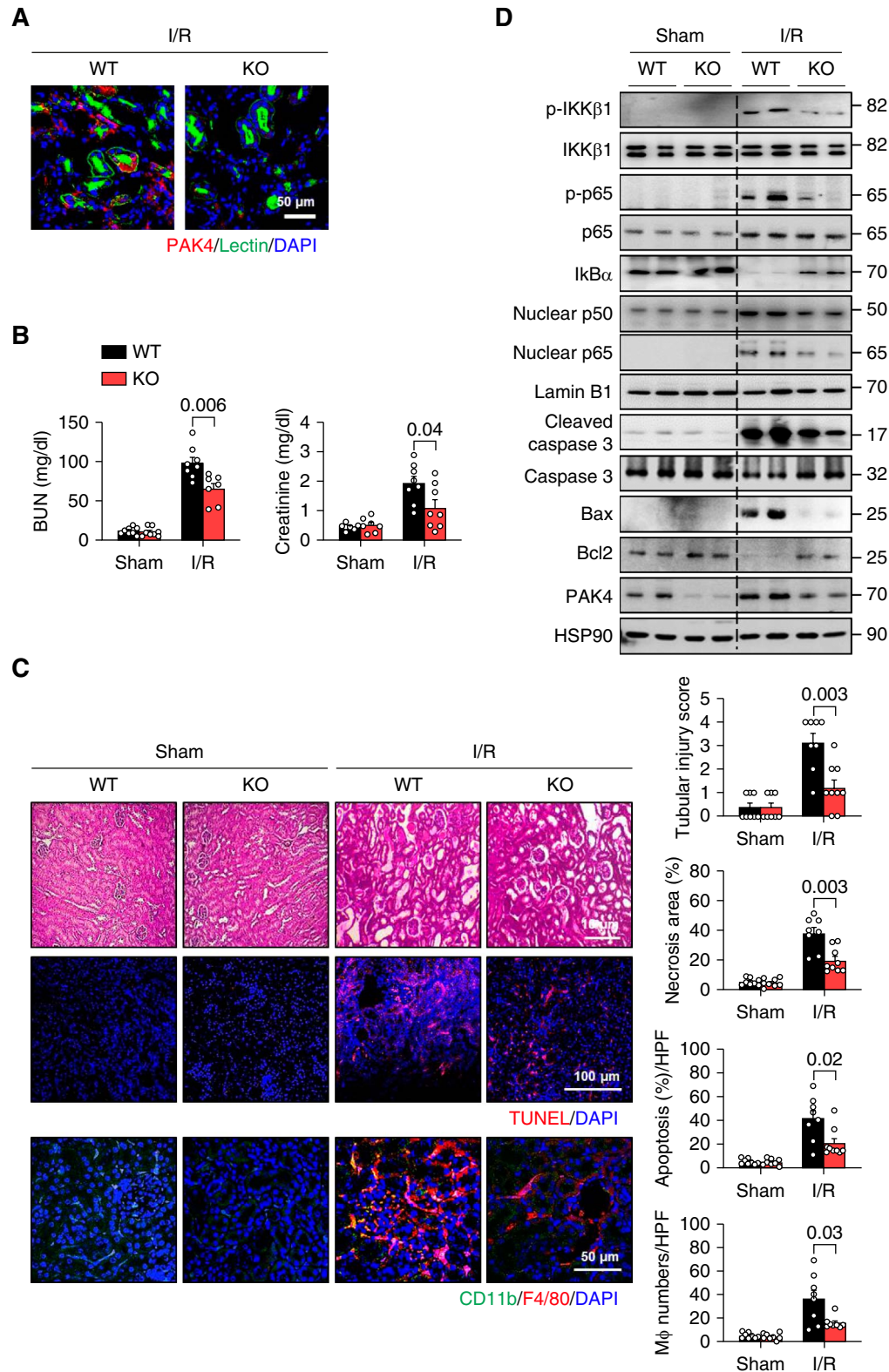


Figure 2. Attenuation of I/R-induced tubular damage and inflammation in *Pak4* KO mice. (A) Immunofluorescence staining of PAK4 and lectin in WT and *Pak4* KO kidney tissues after I/R. (B) Plasma levels of BUN and creatinine ($n=6-9$). (C) Microscopic pictures of kidney sections and quantification of tubular injury score (from H&E), necrosis area (from H&E), apoptosis (from TUNEL), and MΦ numbers (from F4/80) in kidney tissues ($n=8$ or 9). (D) Western blot analysis of apoptosis signaling and inflammation marker molecules in kidney tissues after 24-hour reperfusion. Lamin B1 served as the loading control for nuclear proteins. Values are mean $\pm$ SD. H&E, hematoxylin and eosin; HPF, high-power field; KO, knockout; MΦ, macrophage; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling.

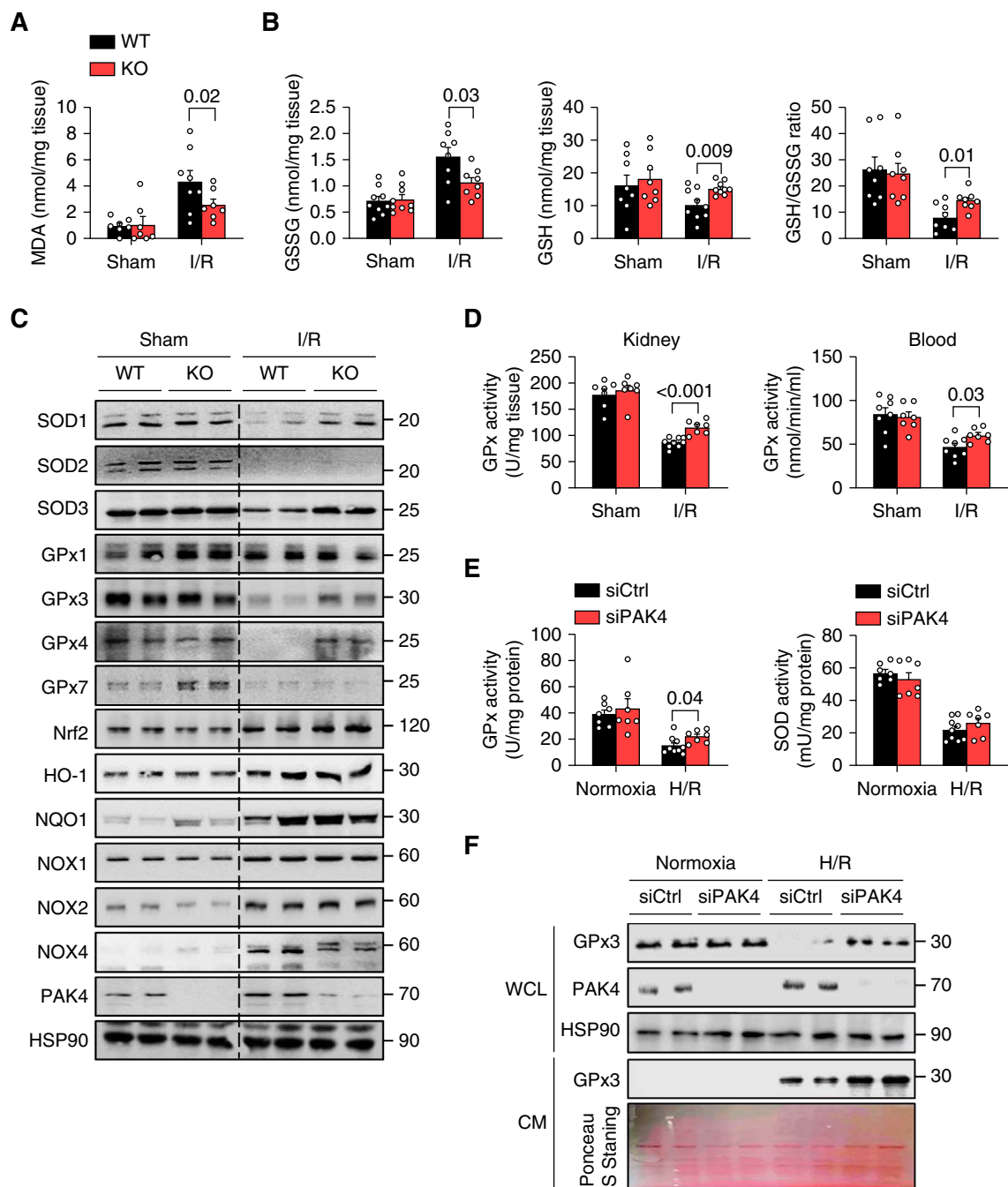


Figure 3. Effects of PAK4 on oxidative stress after renal I/R injury. (A and B) *Pak4* KO and WT littermate control mice were subjected to renal I/R injury. After 24-hour reperfusion, tissue levels of MDA, GSSG and GSH, and the GSH/GSSG ratio were analyzed ($n=7-9$). (C) Western blotting of PAK4 and several antioxidant proteins in kidney tissue homogenates after 24-hour reperfusion. (D) GPx activity in kidney tissues and blood was analyzed ($n=7-9$). (E and F) After silencing PAK4 in HK-2 cells, the cells were cultured in anaerobic jars for 1 hour and reoxygenated for 12 hours. (E) GPx and SOD activities in HK-2 cells were analyzed ($n=7-9$). (F) Western blotting of GPx3 was performed in WCL and CM. Values are mean $\pm$ SD. CM, culture medium; GPx, GSH peroxidase; GPx3, GSH peroxidase 3; GSSG, GSH disulfide; HO-1, heme oxygenase 1; H/R, hypoxia-reoxygenation; MDA, malondialdehyde; NOX, NADPH oxidase; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; WCL, whole cell lysates.

demonstrating that PRKN functions as an E3 ubiquitin ligase to target GPx3 for degradation. *In vitro*, transfection of GPx3^{T47D}, but not GPx3^{T47A}, decreased cellular GPx3 protein level and suppressed GPx3 secretion into the culture medium (Supplemental Figure 8, B and C).

The effects of GPx3^{WT} and GPx3^{T47A} on AKI were further investigated in mice. *Ggt1-Cre* mice were intravenously injected with AAV2 that expressed either the GPx3^{WT} or GPx3^{T47A} (Supplemental Figure 8D). Renal tubule-specific delivery of GPx3 was confirmed by qPCR

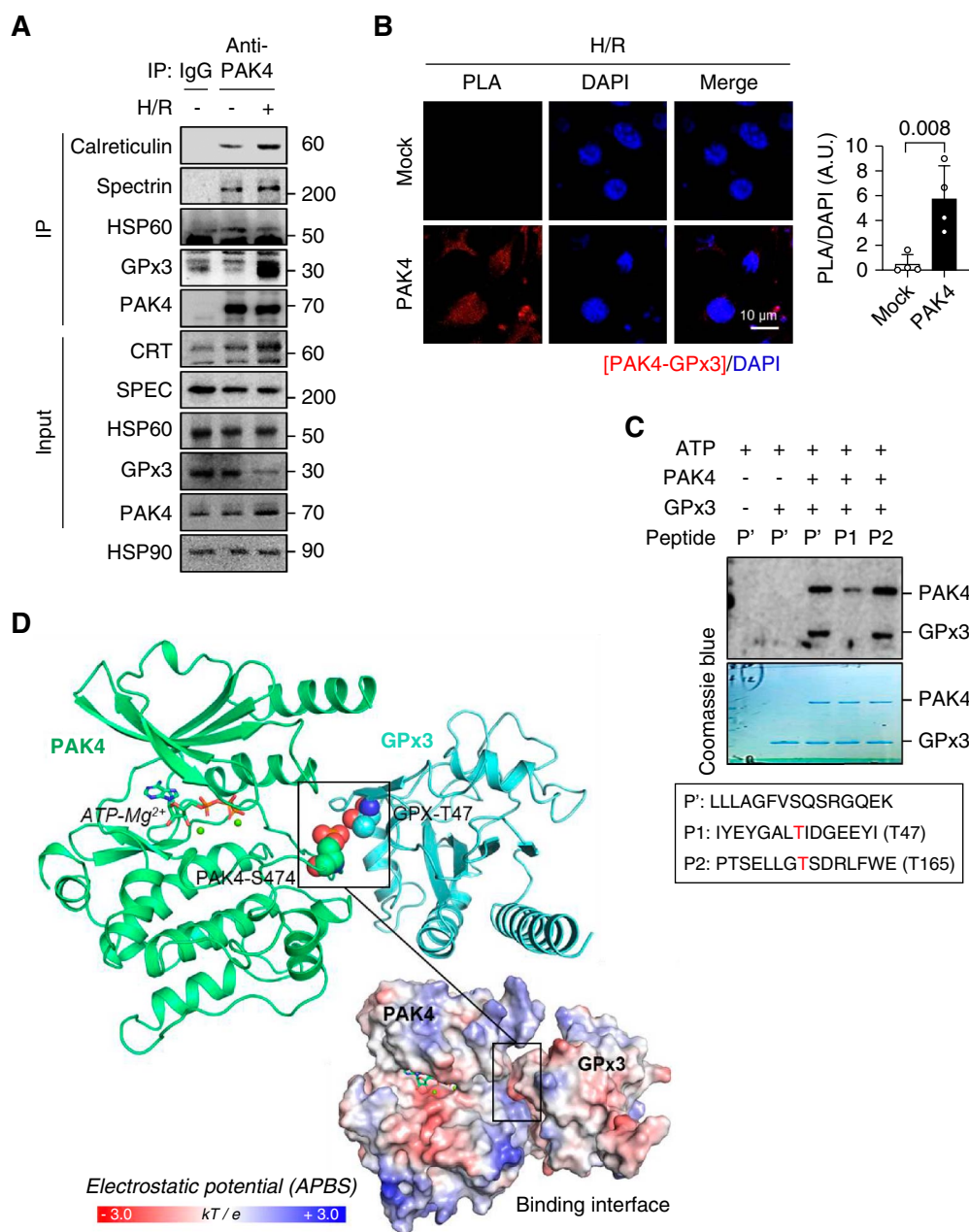


Figure 4. Phosphorylation of GPx3 at T47 by PAK4. (A) After H/R, HK-2 cell lysates were subjected to co-immunoprecipitation to determine PAK4's interaction with and phosphorylation of PAK4 substrate candidates. (B) HK-2 cells were transfected with PAK4 and then exposed to H/R, after which PAK4 binding to GPx3 was analyzed by PLA ($n=4$). (C) *In vitro* kinase assay was conducted with or without the inclusion of 10 μ g of each oligopeptide. Specifically, P' represented the nonspecific negative peptide, whereas P1 denoted the peptide encompassing the T47 region and P2 indicated the peptide encompassing the T165 region. (D) Predicted model of the interaction between PAK4 and GPx3, represented by green and light blue, respectively, in their ternary structure. Values are mean $\pm$ SD. A.U., arbitrary units; IP, immunoprecipitation; PLA, proximity ligation assay.

and immunohistochemistry (Supplemental Figure 8, E and F). Similar to previous results, I/R insult increased kidney tissue injury in *Ggt1-Cre* mice harboring GPx3^{WT} (Figure 5C and Supplemental Figure 8G). However, injection of AAV2-GPx3^{T47A} markedly attenuated these changes, as evidenced by decreases in apoptosis, inflammation, and oxidative stress in kidney tissues and BUN and creatinine levels in the plasma.

PAK4 Deficiency Increased Fatty Acid β -Oxidation in Kidney Tissues

Because the preservation of redox equilibrium is intricately linked to mitochondrial fatty acid β -oxidation⁸ and PAK4 has been shown to inhibit fatty acid β -oxidation in hepatocytes,²³ we next questioned whether the renoprotective effects of PAK4 deficiency is related to the fatty acid metabolism in the renal tubular cells. Biochemical

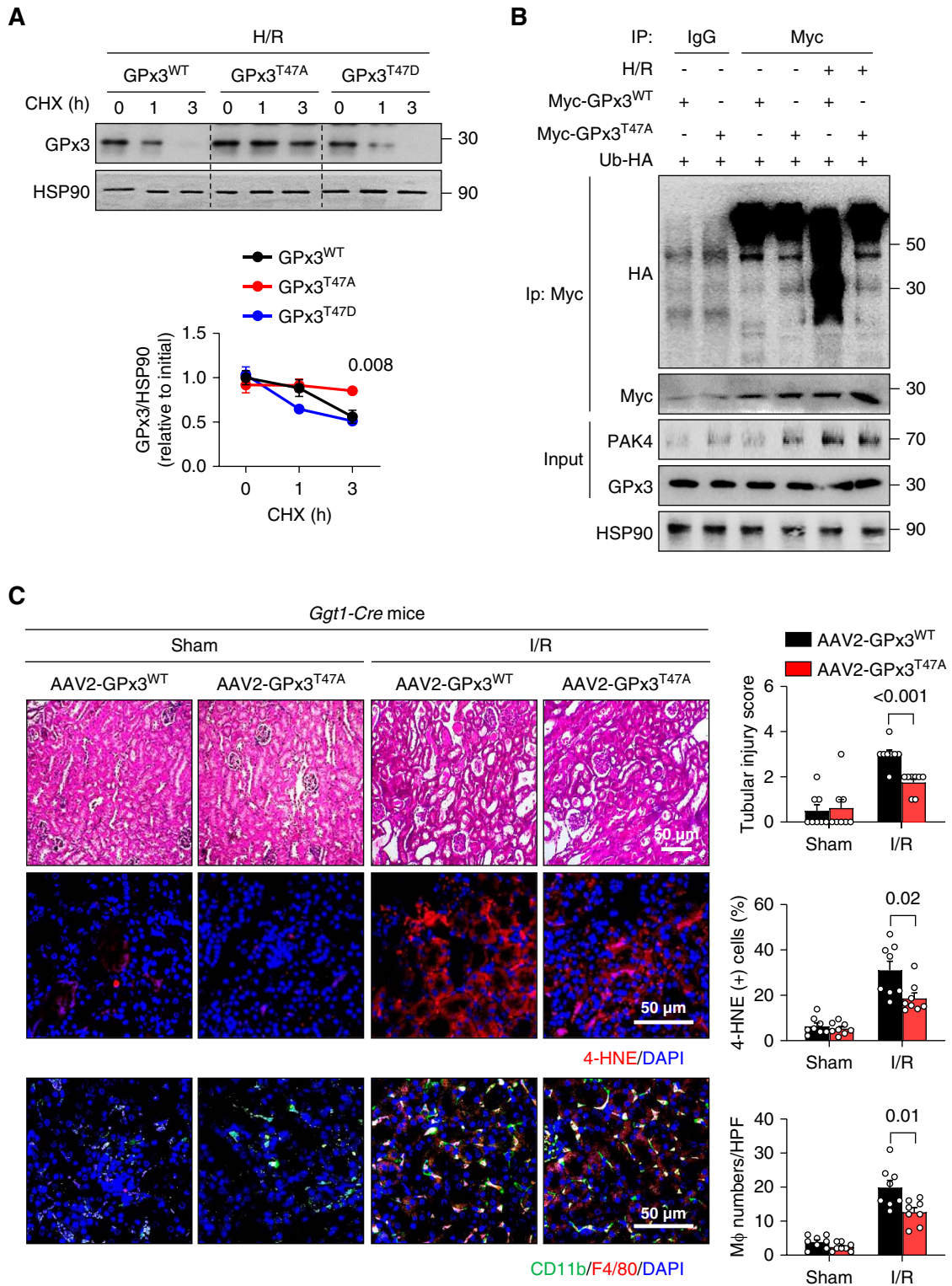


Figure 5. Proteasome-dependent degradation of GPx3 by PAK4. (A) HK-2 cells were transfected with PAK4 and either WT or mutant GPx3 and then treated with CHX (30 μ g/ml) for 1 or 3 hours. The relative protein levels of GPx3 were compared ($n=5$). (B) HK-2 cells transfected with hemagglutinin-tagged ubiquitin (HA-Ub) were transfected with WT (GPx3^{WT}) or phospho-deficient GPx3 (GPx3^{T47A}). Cells were exposed to H/R for 12 hours with MG132 (3 μ M). Cell lysates were immunoprecipitated with anti-GPx3 antibody followed by immunoblotting with anti-Ub antibody. (C) 8-week-old male *Ggt1-Cre* KO mice were injected intravenously with WT GPx3 (AAV2-GPx3^{WT}) or GPx3^{T47A} (AAV2-GPx3^{T47A}). After 24-hour reperfusion, I/R-induced tissue injury was determined. Values are mean $\pm$ SD. 4-HNE, 4-hydroxynonenal; CHX, cycloheximide; Ub, ubiquitin.

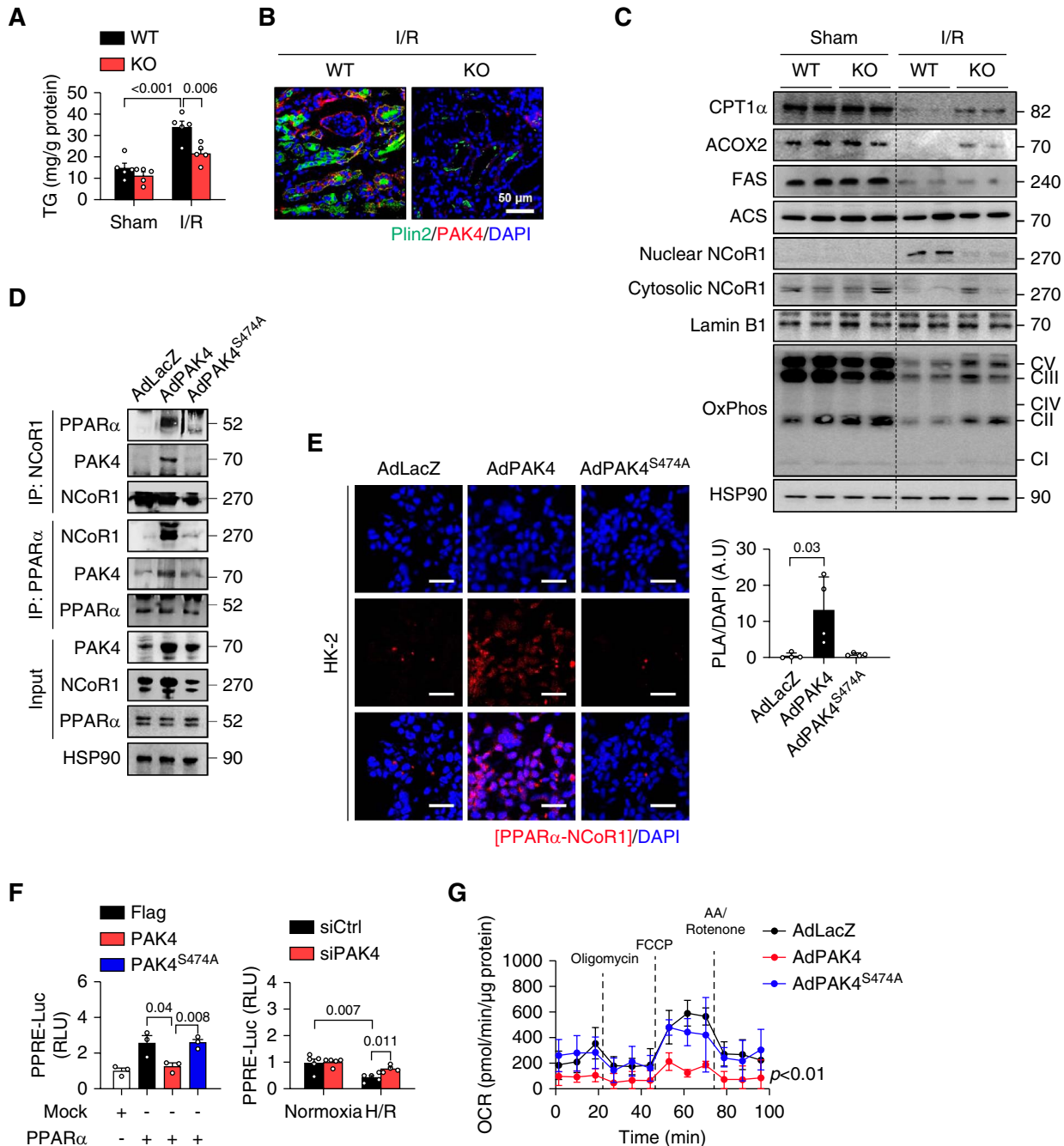


Figure 6. Suppression of fatty acid β -oxidation in kidney by PAK4. (A) After 24-hour reperfusion, TG levels in kidney tissues were analyzed ($n=5$). (B) Plin2 immunostaining was performed to determine fat accumulation in kidney tissues. (C) Western blotting analysis of fatty acid β -oxidation (CPT1 α and ACOX2) and *de novo* lipogenesis-related proteins (FAS and ACS). (D and E) HK-2 cells were infected with the adenoviruses expressing control (AdLacZ), PAK4 (AdPAK4), or kinase-inactive mutant PAK4^{S474A} (AdPAK4^{S474A}), as indicated. (D) Cell lysates were immunoprecipitated with the antibodies against NCoR1 or PPAR α , followed by immunoblotting with anti-PAK4, anti-PPAR α , or anti-NCoR1 antibodies. (E) PPAR α binding to NCoR1 was analyzed with a PLA. Red fluorescent spots indicate that PPAR α and NCoR1 are closely interacting. (F) After overexpressing or silencing PAK4 in primary tubular cells, PPRE-luciferase activities were measured and expressed as the fold change relative to mock or normoxia ($n=3$ or 5). (G) Oxygen consumption rate (OCR) in primary tubular cells was determined using the Seahorse analysis system ($n=5$). Values are mean $\pm$ SD. AA, antimycin A; ACO, acetyl CoA oxidase; ACS, acetyl CoA synthetase; CPT1, carnitine palmitoyltransferase 1; FAS, fatty acid synthase; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; NCoR1, nuclear receptor corepressor 1; OxPhos, oxidative phosphorylation; Plin2, perilipin; PPAR α , peroxisome proliferator-activated receptor alpha; PPRE, PPAR-response element; RLU, relative light units; TG, triglyceride.

triglyceride analysis and perilipin immunostaining results demonstrated that renal steatosis on I/R insult was alleviated by PAK4 deficiency (Figure 6, A and B). PAK4

deficiency led to enhanced fatty acid β -oxidation in kidney tissues, as evidenced by increased protein levels of carnitine palmitoyltransferase 1 and acetyl CoA oxidase

compared with WT mice (Figure 6C). In conjunction with increased mitochondrial fatty acid β -oxidation in *Pak4* KO mice, a significant increase in mitochondrial electron transport chain proteins was observed (Figure 6C). The effect of PAK4 deficiency on fatty acid β -oxidation was also observed in *in vitro* cell culture models. Overexpression of PAK4, but not of PAK4^{S474A}, increased the phosphorylation of nuclear receptor corepressor 1 (NCoR1) and the interaction between peroxisome proliferator-activated receptor alpha (PPAR α) and NCoR1 (Figure 6, D and E). Consequently, this event led to suppressed PPAR α luciferase activity and mitochondrial oxygen consumption in renal tubular cells, while PAK4 silencing augmented PPAR α luciferase activity (Figure 6, F and G).

Pharmacologic Degradation of PAK4 Mitigated Renal I/R Injury in Mice

PROTACs have recently emerged as a therapeutic modality for various diseases. These small molecules consist of three components: a ligand targeting the protein for degradation, an E3 ubiquitin ligase-recruiting ligand, and a linker connecting the two. By facilitating complex formation, PROTACs promote ubiquitination and proteasomal degradation of the target protein. We developed a PAK4 PROTAC, SJ-05, using the PAK4 chemical inhibitor ND201651²⁰ as the warhead (PAK4 ligand). SJ-05 consists of a PAK4 binder, an optimized chemical linker, and the E3 ligase cereblon (CRBN) (Figure 7A). We evaluated its efficacy in protecting against renal I/R injury and demonstrated its promising oral pharmacokinetic characteristics in mice (Figure 7B, Supplemental Figure 9A, and Supplemental Table 2). Consistent with the results observed in *Pak4* KO mice, administration of SJ-05 markedly diminished I/R injury (Figure 7C and Supplemental Figure 9, B–E), effects that were absent in *Pak4* KO mice (Supplemental Figure 10), ruling out the possibility of off-target effects. In addition, SJ-05 was well-tolerated, as no tissue damage was observed in the liver, heart, or kidneys of SJ-05-treated mice compared with control mice (Supplemental Figure 11).

Finally, to understand SJ-05's mode of action, we predicted the ternary complex structure of PAK4, CRBN, and SJ-05 (Figure 7D). The model indicated that SJ-05's linker bridges the thalidomide binding domain of CRBN and the PAK4 kinase hinge region.

PAK4 Expression in Kidney Tissues after Kidney Transplantation Showed an Inverse Correlation with Kidney Function

We examined kidney tissues from individuals who underwent kidney transplantation to explore the relationship between PAK4 expression and the extent of transplantation outcomes. In line with findings from animal studies, individuals exhibiting higher PAK4 protein expression in the kidney cortex also displayed elevated levels of p-GPx3-T47 compared with those with lower PAK4 protein expression (Figure 8A). Importantly, the degree of PAK4 expression and GPx3 phosphorylation in the kidney cortex exhibited an inverse correlation with transplantation outcomes (BUN and creatinine levels) on postoperative day 1 (Figure 8B), offering additional

support for the role of PAK4 in regulating oxidative stress in the human kidney.

Discussion

This research underscores the significance of PAK4 in the I/R process within kidney tissue, building on a previous investigation conducted in the liver.²⁰ In both cases, the upregulation of PAK4 in I/R models proves detrimental. Intriguingly, the mechanism of this effect differs between hepatocytes and renal tubule cells. In hepatocytes, PAK4 plays a critical role in determining the subcellular localization of Nrf2, thereby influencing its transcriptional activity, while in renal tubule cells, PAK4 is essential for the destabilization of GPx3. Consequently, PAK4 seems to regulate oxidative stress in a cell-specific manner, potentially dependent on distinct phosphorylation substrates expressed in both cell types.

The accumulation of ROS during the I/R process is crucial in the development of AKI.¹ After reperfusion, the initial injury event involves mitochondria-producing ROS, leading to secondary tissue damage and subsequent inflammation caused by nonmitochondrial ROS.²⁴ Key defense mechanisms against oxidative stress include Nrf2, SOD, and GPx.¹² In the pursuit of identifying PAK4's phosphorylation substrate among these candidates, Western blotting analysis revealed minimal alterations in Nrf2 and its downstream targets, SOD 2, GPx1, GPx4, or GPx7 protein levels due to PAK4 deficiency, whereas a notable increase in GPx3 was observed in *Pak4* KO mice. Furthermore, co-immunoprecipitation analysis showed a significant interaction between PAK4 and GPx3, suggesting PAK4's selective phosphorylation of GPx3. GPx3 is primarily synthesized in proximal renal tubule cells and exclusively resides in the extracellular compartment. A recent study has emphasized a positive correlation between reduced GPx3 activity and renal oxidative stress in humans,²⁵ underscoring the crucial involvement of kidney tissue GPx3 in AKI pathogenesis. Nonetheless, the regulatory mechanisms governing renal GPx3, particularly in the context of AKI, remain incompletely understood. Herein, we present evidence that PAK4-mediated phosphorylation of GPx3 at T47 leads to GPx3 destabilization. Specifically, on PAK4-mediated phosphorylation of GPx3-T47, PRKN acts as an E3 ubiquitin ligase, targeting GPx3 for ubiquitination and subsequent proteasomal degradation. This finding is supported by the significant elevation of both PAK4 and p-GPx3-T47 in human post-transplant kidney biopsy tissues and mouse kidney tissues after I/R injury. In addition, kidney tissue PAK4 protein levels exhibit a positive correlation with kidney injury markers, emphasizing the importance of the kidney tissue PAK4-GPx3 axis in AKI pathogenesis. Considering its prominent localization in the systemic circulation, it is imperative to investigate whether PAK4 modulates the release of GPx3 into the extracellular compartment and whether extracellular GPx3 exerts a protective function against AKI. Furthermore, additional studies are required to determine whether GPx3 phosphorylation at T47 influences its enzymatic activity.

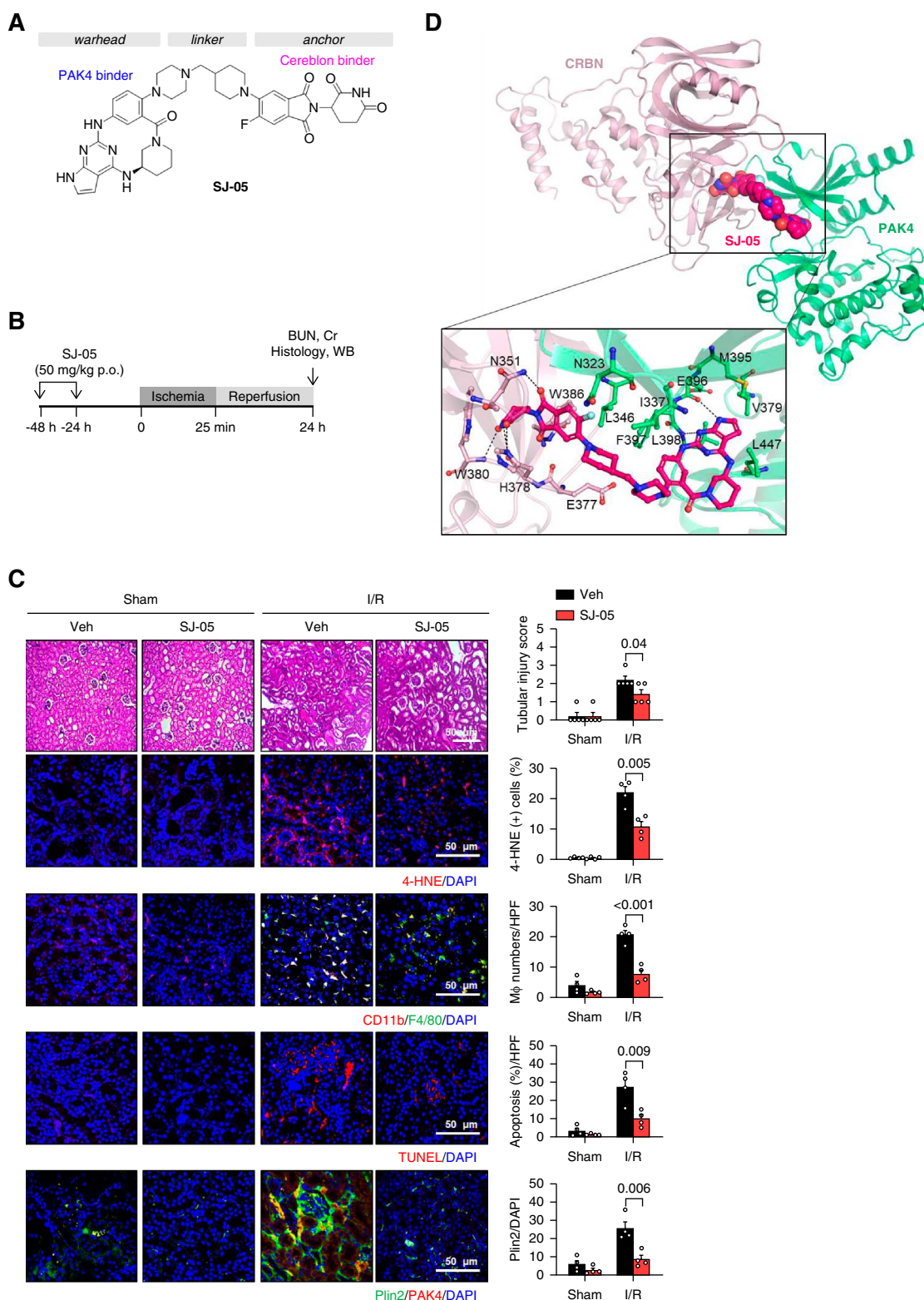


Figure 7. Attenuation of renal I/R injury by PAK4 PROTAC SJ-05. (A) Chemical structure of SJ-05. (B) Schematic diagram of treatment of SJ-05 to C57BL/6 mice. (C) Microscopic pictures of kidney sections and quantification of renal tubular injury ($n=4-5$). (D) Tertiary complex model of PAK4, CRBN, and the PROTAC SJ-05. The secondary structures of PAK4 and CRBN are colored green and light pink, respectively, with the bound SJ-05 molecule represented as magenta spheres. The lower panel provides a close-up view of the binding interface, illustrating the detailed interactions between SJ-05 and the key residues of PAK4 and CRBN. The key interacting residues are represented in sticks, and H-bonding interactions are depicted with black dashed lines. Hydrogen atoms are undisplayed for visual convenience. Values are mean $\pm$ SD. PROTAC, proteolysis-targeting chimera; Veh, vehicle; WT, Western blot.

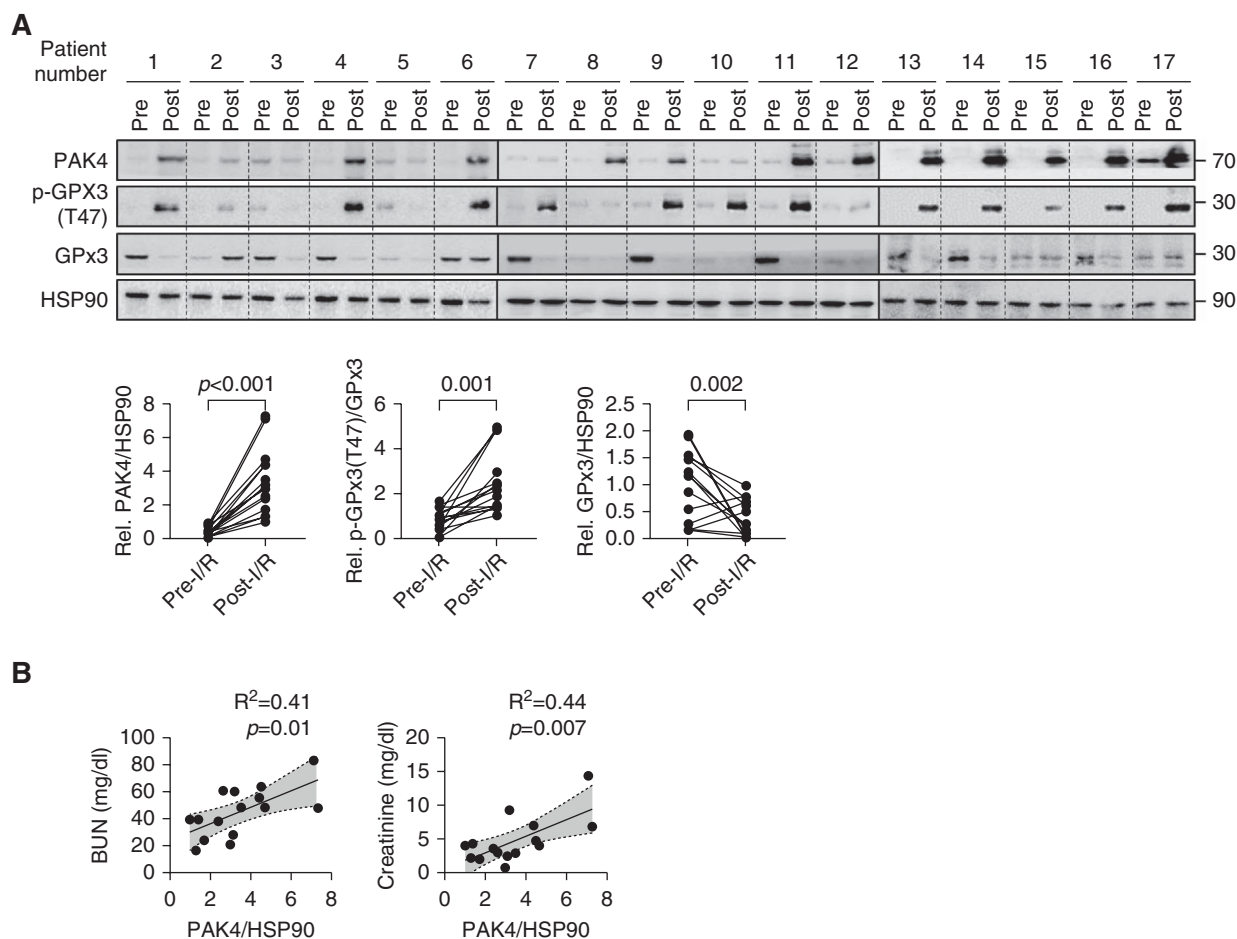


Figure 8. Relationship between PAK4 expression in kidney tissues and transplantation outcomes in humans. (A) Representative Western blot analysis of PAK4 and p-GPx3-T47 in human kidney tissues. Protein density was quantified ($n=15$). (B) Correlation between the PAK4 protein levels (PAK4/HSP90) and the plasma levels of BUN and creatinine on postoperative day 1 ($n=15$). Values are mean $\pm$ SD. p-GPx3 (T47), phospho-GPx3 at T47; Post, postperfusion after kidney transplantation; Pre, preperfusion before kidney transplantation.

During hypoxia or ischemic conditions, fatty acid β -oxidation is restricted and mitochondrial respiration is compromised.⁵ Consequently, the formation of lipid droplets becomes a common occurrence in proximal tubule cells during AKI. PPAR α , predominantly expressed in proximal tubules and scarcely in other kidney segments,²⁶ plays a pivotal role in regulating fatty acid β -oxidation. Targeting the impaired fatty acid metabolism through PPAR α activation has been demonstrated to confer renoprotection in AKI scenarios.^{27,28} In addition, the identification of coactivators, corepressors, or endogenous PPAR α ligands in the proximal tubule cells would help to elucidate the molecular mechanisms by which I/R leads to the downregulation of fatty acid β -oxidation enzymes. Our recent findings have demonstrated that PAK4 phosphorylates NCoR1, a corepressor of PPAR α , to inhibit PPAR α transcriptional activity in hepatocytes.²³ Hepatocyte-specific *Pak4* KO mice exhibit diminished expression of genes associated with fatty acid β -oxidation and ketogenesis on fasting, accompanied by the suppression of NCoR1 nuclear transport. Similar to these findings, in this study, mice with proximal tubule-specific PAK4 deficiency exhibit reduced triglyceride accumulation alongside enhanced

expression of fatty acid β -oxidation genes. In renal tubular cells, PAK4 also regulates the nuclear transport of NCoR1 and its interaction with PPAR α . These findings highlight the PAK4-NCoR1-PPAR α axis as a promising therapeutic target to overcome AKI.

In this study, we developed a small molecule-based PAK4 PROTAC using a hybrid molecule approach, marking the first orally available PAK4 protein degrader. Our strategy used ND201651, which exhibited selectivity for PAK4 (IC_{50} 4.22 nM) over PAK1 (IC_{50} 1200 nM), as a warhead.²⁰ This entailed conjugation of the small-molecule PAK4 inhibitor ND201651²⁰ with a ligand specific to the CRBN E3 ubiquitin ligase, facilitating targeted degradation of PAK4. Presently, small molecules are predominantly used as targeting warheads in PROTAC design. Unlike small-molecule inhibitors, PROTACs possess the ability to bind the target at various positions, including nonfunctional binding sites.²⁹ Given the high sequence homology among PAK family members and the challenges in achieving complete target inhibition with conventional inhibitors, we aimed to develop a PROTAC-based approach for selective PAK4 degradation.³⁰ PROTACs offer an advantage over inhibitors by exploiting protein

degradation, offering greater selectivity for the target protein compared with the binding profile of the parent warhead.³¹

While preparing our manuscript, a peptide-based PAK4 PROTAC, shown to be effective through abdominal injection in treating renal cell carcinoma in mouse models and patient-derived organoids, was reported.³² Despite the limitations associated with small-molecule-based PROTACs, such as challenging chemical synthesis, off-target toxicity, poor oral absorption, and limited transmembrane properties, we successfully addressed these issues with SJ-05 by using a molecular design and synthetic strategy. Leveraging computational modeling techniques, we optimized the structure of PAK4-targeting PROTACs on the basis of ND201651 to retain its binding affinity toward PAK4 and to improve the oral absorption and cellular permeability. This resulted in the development of SJ-05, which exhibited a favorable oral pharmacokinetic profile. SJ-05's efficacy depends on PAK4 levels, which are low in normal kidneys but elevated in I/R-injured tissues, minimizing its effect on normal tissue. These findings collectively highlight the potential benefits of PAK4 PROTAC treatment for patients with AKI.

Beyond the realm of PAK4 biology, this study carries clinical significance, offering potential avenues for the development of new therapies for acute kidney disease—a global public health concern, with high morbidity and mortality.³³ Notably, the nearly complete reversal of I/R-induced AKI by inhibiting the PAK4 and GPx3 axis using PAK4 PROTAC underscores PAK4 as a promising therapeutic target for renal I/R injury.

Disclosures

Disclosure forms, as provided by each author, are available with the online version of the article at <http://links.lww.com/JSN/F91>.

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Data Sharing Statement

All data are included in the manuscript and/or supporting information. Partial restrictions to the data and/or materials apply. The datasets used and/or analyzed during the current study are accessible from the corresponding author on reasonable request.

Supplemental Material

This article contains the following supplemental material online at <http://links.lww.com/JSN/F92>.

Supplemental Methods

Supplemental Figure 1. Generation of renal tubule-specific *Pak4* KO mice.

Supplemental Figure 2. Attenuation of cisplatin-induced tubular damage and inflammation in *Pak4* KO mice.

Supplemental Figure 3. No effects of myeloid PAK4 deficiency on renal I/R injury.

Supplemental Figure 4. Protein quantification.

Supplemental Figure 5. Phosphoproteomic analysis of kidney tissues.

Supplemental Figure 6. Effects of PAK4 on GPx3 transcription.

Supplemental Figure 7. Binding and phosphorylation of GPx3 by PAK4.

Supplemental Figure 8. Destabilization of GPx3 by PAK4.

Supplemental Figure 9. Attenuation of renal I/R injury by PAK4 PROTAC SJ-05.

Supplemental Figure 10. On-target effects of SJ-05 treatment.

Supplemental Figure 11. No potential acute side effects of SJ-05.

Supplemental Table 1. Calculated stoichiometry of phosphorylation based on label-free LC/MS data.

Supplemental Table 2. Pharmacokinetic parameters of SJ-05 following oral doses of 10 mg/kg in mice.

Supplemental Table 3. Clinical factors and graft outcome indicators after transplantation.

Supplemental Table 4. Antibodies used for Western blotting, immunofluorescence, and immunohistochemical analyses.

Supplemental Table 5. Sequences and accession numbers for primers (forward, FOR; reverse, REV) used in qPCR.

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