PRDM16 deficiency triggered by TGF-β signaling aggravated renal fibrosis by promoting tubular mitochondrial dysfunction

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Article

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Abstract

TGF-β signaling is the master modulator of renal fibrosis. However, its targeting drugs are failed to prevent the progression of chronic kidney disease (CKD) in clinical trials due to the extensive biological regulation of TGF-β signaling. It is necessary to investigate the precise downstream of TGF-β signaling that regulates renal fibrosis. In this study, we found that transcription factor PRDM16 expressed by human renal tubular epithelial cells was reduced markedly by TGF-β. Mechanistically, the activated Smad3 induced by TGF-β interacted with the cofactor, H-Ras, and bound to the promoter of PRDM16 to downregulate its transcription. Tubular-specific knockout of PRDM16 promoted renal fibrosis of unilateral ureteral occlusion (UUO) and unilateral ischemia-reperfusion injury (UIRI) mice by aggravating mitochondrial dysfunction. \textit{In vitro}, PRDM16 blocked TGF-β-induced mitochondrial injury, and lipid deposition by upregulating PGC-1α. PRDM16 supplementary therapy preserved renal function and ameliorated the progression of renal fibrosis by protecting mitochondrial function. We report a novel downstream of TGF-β signaling, PRDM16 for attenuating renal fibrosis by protecting tubular mitochondrial function.

Introduction

Chronic kidney disease (CKD) is defined as decreased renal function or abnormal markers of kidney damage caused by various reasons for more than 3 months\(^1\). CKD has become a worldwide public health problem with a high prevalence which is 10–13% in adults and high mortality due to the progression into end-stage renal disease (ESRD)\(^2\). Renal fibrosis which is characterized by tubular atrophy, immune cells and myofibroblasts infiltration, and extracellular matrix (ECM) deposition is the common pathology of CKD and a key accelerator of the progression of CKD to ESRD\(^3\)–\(^5\). Thus, attenuating renal fibrosis is an effective approach to decreasing the number of ESRD patients. However, the therapies for ameliorating renal fibrosis are limited.

Transforming growth factor β (TGF-β) signaling is a developmental pathway that regulates multiple normal physiological processes such as cell growth and differentiation, autophagy, and the immune response\(^6\). In the canonical TGF-β signaling cascade, TGF-β1 binds to the TGF-β receptor dimer and activates Smad2 and Smad3. Phosphorylated Smad2/3 translocates into the nucleus to regulate downstream targets. TGF-β signaling is extensively studied in the pathogenesis of organ fibrosis\(^7\). And it is a master regulator of renal fibrosis that modulates fibroblasts activation and proliferation, tubular injury through partial EMT and energy metabolism disorder, endothelial-to-myofibroblast transition, and macrophage-to-myofibroblast transition\(^8\). Due to its strong role in renal fibrosis, TGF-β signaling-targeted drugs are widely investigated and display encouraging anti-fibrotic effects in pre-clinical animal studies\(^9\). Pirfenidone (NCT00063583), LY2382770 (NCT01113801), and Fresolimumab (NCT01665391) are the most striking potential candidates in the application into patients with renal fibrosis\(^10\). However, the results from clinical trials of those drugs are disappointing\(^11,12\). We notice that those drugs blunt the interaction between TGF-β and TGF-β receptor, blocking the entire TGF-β signal cascade which regulates
many biological responses besides fibrosis. Thus, exploring precise downstream of TGF-β signaling that regulates renal fibrosis is necessary.

The PRDF1 and RIZ1 homology domain (PRDM) protein family contains 17 members who share the common conserved N-terminal PR domain and a variable number of zinc fingers (ZNF)\textsuperscript{13}. PRDM proteins mediate histone methylation due to the similarity of the PR domain to the SET domain which resides on histone lysine methyltransferases (HMTs). The ZNF domain of PRDM binds to DNA and regulates the transcription of target genes\textsuperscript{14}. PRDM proteins participate in the regulation of various diseases including lymphoma, leukemia, cardiomyopathy, and metabolism disorder\textsuperscript{13}. However, the role of the PRDM family in kidney disease remains unclear.

We detected the expression of the PRDM family in human tubular epithelial cells treated with TGF-β\textsubscript{1}. And we found that PRDM16 decreased most greatly after TGF-β\textsubscript{1} treatment. And PRDM16 decreased markedly in the fibrotic kidney of mice and humans compared to healthy control. Phosphorylated Smad3 activated by TGF-β decreased the transcription of PRDM16 by binding to the promoter of PRDM16. And H-Ras acted as the transcription coactivator of p-Smad3 to regulate the expression of PRDM16. Tubular-specific knockout PRDM16 aggravated renal fibrosis by promoting mitochondrial dysfunction in the kidney of UUO and UIRI mice. However, PRDM16 overexpression blocked TGF-β induced mitochondrial injury, lipid deposition, and ECM production in human tubular epithelial cells by upregulating the expression of PGC-1α. Lastly, we demonstrated that PRDM16 supplementary therapy ameliorated mitochondrial dysfunction and renal fibrosis caused by UUO and UIRI surgery. These results suggest that PRDM16 is a precise downstream of TGF-β signaling that mediates renal fibrosis by improving tubular mitochondrial function.

**Result**

**PRDM16 was decreased in the injured kidneys of mice and humans.**

We conducted RNA sequence on human renal tubular epithelial cells treated with TGF-β (Fig. S1a-b). The results showed that the expressions of *PRDM16* decreased most greatly among PRDM family members after TGF-β treatment (Fig. 1a). Then, we analyzed the RNA-sequence data derived from the tubulointerstitial compartment of kidney biopsies of CKD patients on the Nephroseq database\textsuperscript{15}. The result showed that PRDM16 expression was decreased in CKD patients (Fig. 1b). The kidney paraffin biopsies of IgA patients with different stages of tubular atrophy/interstitial fibrosis (T) according to oxford classification were collected, and immunohistochemical staining showed the expression of PRDM16 decreased with the progression of fibrosis (Fig. 1c, 1d). And the expression of PRDM16 was negatively correlated with blood urea nitrogen (Fig. 1e). Then, the PRDM family in mice kidneys was detected. Consistently, PRDM16 was decreased greatly among the PRDM family in the UUO kidney (Fig. S1c). And the expression of PRDM16 was abundant in normal kidneys when compared to the other organs including heart, lung, spleen, and liver (Fig. S1d). The results of Western blotting and qRT-PCR showed that the levels of PRDM16 were reduced in the UUO kidney and the UIRI kidney (Fig. 1f-k).
Immunohistochemical staining showed that PRDM16 was mainly expressed in healthy tubular epithelial cells, but not in UUO tubules (Fig. 1l). Together, PRDM16 was diminished markedly in the fibrotic kidney of humans and mice.

**TGF-β transcriptionally downregulated PRDM16 in an H-Ras/p-Smad3-dependent way.**

We investigated the mechanism of the regulation of TGF-β on PRDM16. Western blotting results showed that TGF-β decreased the protein level of PRDM16 greatly (Fig. 2a, 2b). As illustrated by qRT-PCR, TGF-β transcriptionally downregulated the level of PRDM16 (Fig. 2c). SIS3, which specifically inhibited the phosphorylation of Smad3 was used to demonstrate the role of the transcription factor of canonical TGF-β signaling, Smad3 on the regulation of PRDM16. As expected, p-Smad3 inhibition reversed the decrease of protein and mRNA of PRDM16 caused by TGF-β treatment (Fig. 2d-f). Does the p-Smad3 downregulate PRDM16 by binding to its promoter? We analyzed the JASPAR database and found that there were several DNA-binding elements of Smad3 on the PRDM16 promoter (Fig. 2g). And three primers were designed to recognize these binding elements. Chromatin immunoprecipitation (ChIP) showed PRDM16 promoter could be detected with three primers in the pull-down complex precipitated by anti-p-Smad3 antibodies (Fig. 2h). These results indicated that p-Smad3 downregulated PRDM16 by directly binding to the promoter of PRDM16. Although Smad3 acts as a transcription factor, the affinity of Smad3 and DNA is weak. Thus, the transcription coactivator is necessary for the high-affinity and high-specificity of Smad3 to the promoter. To further investigate the transcription cofactor of Smad3 that regulated the transcription of PRDM16, we conducted DNA pull-down assay. As shown in Fig. 2i, we treated HK-2 cells with TGF-β and extracted the nuclear proteins. And the nuclear proteins were incubated with streptavidin magnetic beads, and biotin-labeled DNA probes which bound to the promoter of PRDM16 specifically. After repeatedly washing, the pull-down proteins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics. 183 different proteins compared to the negative DNA probe control group were found, and they shared only 5 proteins with the “wound healing” data set (GO:0042060) (Fig. 2j). H-Ras was one of the 5 proteins. The mass spectrum of H-Ras was shown in Fig. 2k. H-Ras is a member of the Ras family which is GTPase. H-Ras knockout inhibited fibroblasts proliferation and motility induced by TGF-β and attenuated renal fibrosis in vivo. We knockdown H-Ras with siRNA to validate the effect of H-Ras on PRDM16 expression (Fig. 2l). Western blotting showed that TGF-β treatment decreased the expression of PRDM16 and PGC-1α, and increased the level of fibronectin, however, H-Ras knockdown blocked these changes (Fig. 2m, 2n). These data suggest that H-Ras plays an important role in TGF-β signaling downstream regulation. By consulting the STRING database, we found that H-Ras interacted with Smad3 (Fig. 2o). Co-immunoprecipitation showed that H-Ras was identified in the immunocomplexes precipitated by anti-p-Smad3 antibodies (Fig. 2p). Collectively, Smad3 activated by TGF-β binds to the promoter of PRDM16 to downregulate its transcription with the assistance of H-Ras.

**Tubular-specific knockout PRDM16 aggravated ischemia-reperfusion induced renal interstitial fibrosis by promoting mitochondrial dysfunction.**
To demonstrate the role of PRDM16 on kidney fibrosis, we generated the tubular-specific knockout PRDM16 mice by hybridizing \textit{PRDM16}^{\text{lox/lox}} mice and \textit{Ksp-Cre} \text{mice}^{19}. Genotyping and immunohistochemical staining results revealed PRDM16 expressed by tubular epithelial cells was knockout successfully (Fig. S2a, S2b). And the 8 weeks aged knockout mice were healthy with normal liver function, kidney function, and blood glucose (Fig. 3a, 3b, Fig. S2c-f). As illustrated by Hematoxylin Eosin (HE) staining of the heart, kidney, perirenal fat, lung, spleen, and liver, the structures of important organs were normal (Fig. S2g-i). Then, we performed UIRI surgery on the transgenic mice. The serum creatine and urea nitrogen aggravated in \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} mice compared to \textit{PRDM16}^{\text{lox/lox}} mice after injury (Fig. 3a, 3b). To seek the expression profile influenced by PRDM16 knockout, we conducted RNA-sequencing with UIRI kidneys from \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} mice and \textit{PRDM16}^{\text{lox/lox}} mice. Volcano plots and heat maps showed the gene expression was quite different in the two groups (Fig. 3c, 3d). As demonstrated by Gene set enrichment analysis (GSEA), compared to \textit{PRDM16}^{\text{lox/lox}} kidney, the top 15 suppressed Gene Ontology (GO) sets of \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} were all related to mitochondrial structure and mitochondrial function, and 7 in top 15 activated GO sets were related to the extracellular matrix (Fig. 3e). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed oxidative phosphorylation ranked first (Fig. 3f). These results indicated that PRDM16 deficiency promoted renal fibrosis of UIRI mice mainly by destroying mitochondrial structure and function. Then, we performed experiments to confirm it. Mitochondrial mass as shown by mitotracker staining in \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} mice was less than \textit{PRDM16}^{\text{lox/lox}} after UIRI surgery (Fig. 3g). Mitochondrial injury impaired fatty acid oxidation and resulted in lipid deposition. Bodipy staining showed that lipid deposition was much more severe after PRDM16 knockout (Fig. 3g). From Western blotting results, the key regulator of mitochondrial biogenesis, PGC-1\(\alpha\) was reduced greatly in UIRI mice. And PRDM16 knockout further downregulated the expression of PGC-1\(\alpha\) (Fig. 3h, 3i). Then, renal interstitial fibrosis was evaluated. The Western blotting of \(\alpha\)-SMA and Masson trichrome-staining (MTS) showed the kidney of \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} mice exhibited much more myofibroblasts accumulation and ECM deposition after UIRI surgery (Fig. 3h, 3j, 3k,3l).

**Tubular-specific knockout PRDM16 promoted tubular mitochondrial injury and interstitial fibrosis in UUO mice.**

To generalize the effect of PRDM16 on renal fibrosis, we operated another commonly used animal model of renal fibrosis, the UUO model. 7 days after UUO surgery, the renal cortex and medulla were compressed and thinned due to hydronephrosis. And renal cortex and medulla were thinner in \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} mice compared to \textit{PRDM16}^{\text{lox/lox}} mice (Fig. 4a). Mitochondrial-related genes like \textit{Acox2}, \textit{Ppargc1a}, and \textit{Tfam} decreased more in the \textit{Ksp-Cre/PRDM16}^{\text{lox/lox}} UUO kidney than in the \textit{PRDM16}^{\text{lox/lox}} UUO kidney (Fig. 4b). Transmission electron microscope showed that the mitochondrial loss and cristae vague were aggravated after PRDM16 knockout (Fig. 4c). The expression of PGC-1\(\alpha\) was inhibited in the UUO kidney, and knockdown of PRDM16 exacerbated its decrease (Fig. 4d, 4e). Then we further assessed renal fibrosis in the UUO model. The protein level of fibronectin and \(\alpha\)-SMA, and mRNA levels of \textit{fibronectin}, \textit{Col1a1}, \textit{Col3a}, and \textit{Vimentin} were increased by UUO surgery and were exaggerated by PRDM16 knockout.
Collagen deposition revealed by Masson trichrome-staining was more severe in \textit{Ksp-Cre/PRDM16^{flox/flox}} UUO mice than in \textit{PRDM16^{flox/flox}} UUO mice (Fig. 4j, 4k). These results suggest that loss of PRDM16 promoted mitochondrial dysfunction and the progression of renal fibrosis.

**PRDM16 inhibited TGF-β induced tubular mitochondrial dysfunction and TGF-β signaling.**

To investigate the reno-protective effect of PRDM16, we screened and obtained the PRDM16 overexpressing HK-2 cell lines with lentivirus (Fig. S3). The PRDM16 overexpressing HK-2 cells were treated with vehicle or TGF-β. The transcription of \textit{TFAM} who is master regulator of mitochondrial biogenesis, and \textit{CPT1A} and \textit{ACOX2} that resided in healthy mitochondria were decreased after TGF-β treatment. And PRDM16 overexpression inhibited the downregulation of \textit{TFAM}, \textit{CPT1A}, and \textit{ACOX2} (Fig. 5a). Western blotting showed that PRDM16 overexpression blocked the reduction of PPAR-γ and CPT1A induced by TGF-β (Fig. 5b-d). As Fig. 5e shows, PRDM16 overexpression inhibited the mitochondrial loss, mitochondrial swell, and lipid accumulation caused by TGF-β treatment. And the ATP production was rescued by PRDM16 overexpression (Fig. 5f). We also detected the effect of PRDM16 on partial EMT of tubular epithelial cells. PRDM16 decreased the protein level of fibronectin and the marker of myofibroblasts, α-smooth actin (α-SMA) (Fig. S4a-c), and the mRNA level of \textit{COL1A1} and \textit{fibronectin} (Fig. S4d, S4e). The effect of PRDM16 on classical and non-classical TGF-β signaling was detected. Western blotting showed that PRDM16 overexpression blocked the phosphorylation of Smad3 and ERK in HK-2 cells treated with TGF-β (Fig. 5g-i). The nuclear translocation of p-Smad3 after TGF-β incubation was inhibited by PRDM16 (Fig. 5j).

**PRDM16 rescued tubular mitochondrial function via upregulating PGC-1α.**

Peroxisome Proliferator-Activated Receptor γ (PPARγ) coactivator-1α (PGC-1α) is a key protein that regulates mitochondrial biosynthesis. It interacts with a variety of transcription factors such as Nuclear Respiratory Factor-1 (NRF-1), NRF-2, Estrogen-Related Receptors (ERR), and PPARγ to promote gene expression related to mitochondrial synthesis, oxidative phosphorylation, and fatty acid oxidation\textsuperscript{20}. Then, we investigated the regulation of PRDM16 on PGC-1α. The immunohistochemical staining with serial section of IgA patients showed that PRDM16 co-stained with PGC-1α (Fig. 6a), and the expression of PRDM16 was positively correlated with PGC-1α (Fig. 6b). We used siRNA to knock down PRDM16 \textit{in vitro}. The protein levels of PGC-1α and PPAR-γ were down-regulated (Fig. 6c-f). PRDM16 overexpression blocked the decrease of PGC-1α induced by TGF-β (Fig. 6g-i), as well as the downregulation of PGC-1α and PPAR-γ caused by hypoxia and reoxygenation injury (Fig. 6j-l). ZLN005 acts as an agonist of PGC-1α via promoting the transcription of PGC-1α by binding to the promoter\textsuperscript{21}. ZLN005 alleviated the decrease of PGC-1α and the increase of fibronectin induced by TGF-β (Fig. S5a, S5b). Mitotracker and Bodipy staining showed that ZLN005 protected tubular epithelial cells from mitochondria loss and lipid accumulation (Fig. S5c). The rescue experiments showed that ZLN005 blunted PRDM16 knockdown induced the decrease of PGC-1α and the increase of fibronectin (Fig. 6m-o). These data indicate that PRDM16 prevents mitochondrial dysfunction \textit{in vitro} by upregulating PGC-1α.
PRDM16 supplement with lentivirus attenuated mitochondrial dysfunction and interstitial fibrosis in the UIRI model.

To explore the renal protective effect of PRDM16 supplementary therapy, we sought to overexpress PRDM16 \textit{in vivo} with lentivirus and then performed ischemia-reperfusion surgery on the injected kidney. Ten days later, we removed another kidney and sacrificed the mice after 24 hours (Fig. 7a). Renal functions reflected by serum creatine and urea nitrogen were increased in UIRI mice, but PRDM16 overexpression reduced their levels (Fig. 7b, 7c). As illustrated in Fig. 7d and e, PRDM16 protein was overexpressed in tubular epithelial cells of UIRI mice. qRT-PCR for \textit{PRDM16} mRNA expression showed similar results (Fig. 7f). The expression of the downstream target of PRDM16, PCG-1α, was restored due to PRDM16 overexpression as demonstrated by immunohistochemical staining (Fig. 7g, 7h), qRT-PCR (Fig. 7i), and Western blotting (Fig. 7j, 7k). As shown in Fig. 7n, mitochondrial mass exhibited by mitotracker staining was significantly decreased in the UIRI kidney. However, treatment with PRDM16 overexpression lentivirus largely preserved mitochondrial mass. The mitochondrial repair facilitated FAO and then protected tubular cells from lipid deposition. Thus, lipid accumulation revealed by Oil-red O staining was alleviated by PRDM16 overexpression (Fig. 7o). We have shown the mitochondrial protective role of PRDM16. Then, we further assessed the effects of PRDM16 on renal interstitial fibrosis. Western blotting showed that overexpression of PRDM16 inhibited renal expression of fibronectin, α-SMA in UIRI mice (Fig. 7j, 7l, 7m). The immunohistochemical staining of α-SMA showed a consistent result (Fig. 7p, 7q). PRDM16 overexpression also attenuated collagen deposition and fibrotic lesions in the kidney, as shown by Masson's trichrome staining (Fig. 7p, 7r). Thus, these data revealed that the PRDM16 supplement is effective to protect the kidneys from chronic injury.

PRDM16 overexpression also ameliorated mitochondrial dysfunction and interstitial fibrosis of UUO mice.

Next, we conducted the UUO model to further confirm the renal protective effect of PRDM16. Mice were intravenously treated with the expression vector encoding PRDM16 through the hydrodynamic-based gene delivery method\textsuperscript{22}. As expected, PRDM16 was dramatically induced in the kidney that even suffered from UUO injury (Fig. 8a-d). The downstream target, PGC-1α was upregulated by PRDM16 overexpression compared to the UUO kidney as revealed by immunohistochemical staining, Western blotting, and qRT-PCR (Fig. 8e-h). Restoration of PGC-1α is beneficial to mitochondrial repair after UUO. Thus, the ATP production was rescued in the PRDM16 overexpression UUO kidney (Fig. 8i). Mitochondrial loss and lipid accumulation were obvious in the UUO kidney, and PRDM16 overexpression ameliorated these lesions (Fig. 8j, 8k). Renal fibrosis was also detected. Collagen deposition as illustrated by Sirius Red staining and Masson's trichrome staining, and fibronectin accumulation as shown by immunohistochemical staining were attenuated by PRDM16 plasmid injection compared to the empty vector group (Fig. 8l-n). The mRNA levels of \textit{fibronectin} and \textit{Col1a1} showed similar results (Fig. 8o, 8p). Generally speaking, PRDM16 overexpression inhibited the progression of mitochondrial dysfunction and renal fibrosis.

Lastly, we summarized the findings of this study (Fig. 9). PRDM16 was decreased markedly in patients and mice with renal fibrosis. p-Smad3 activated by TGF-β transcriptionally downregulated the expression
of PRDM16 by directly binding to the promoter of PRDM16 with the assistance of H-Ras. The decreased PRDM16 augmented the activation of TGF-β signaling and reduced the expression of PGC-1α, resulting in mitochondrial dysfunction and lipid deposition. PRDM16 supplementary therapy attenuated renal fibrosis and mitochondrial dysfunction of UUO and UIRI mice by upregulating PGC-1α.

Discussion

Here, we demonstrated that PRDM16 was a novel downstream of TGF-β signaling that regulated renal fibrosis by protecting tubular mitochondrial function. TGF-β transcriptionally decreased the expression of PRDM16 in the fibrotic kidney in an H-Ras/Smad3-dependent way. PRDM16 supplementary therapy attenuated renal fibrosis and preserved tubular mitochondrial function by upregulating PGC-1α.

The role of the PRDM family has not been studied in kidney diseases. We first examined the expression of PRDM members in human tubular epithelial cells treated with TGF-β. Among them, PRDM16 decreased most greatly. And we noticed that PRDM1 increased markedly after TGF-β treatment. It was reported that PRDM1 knockout in T cells facilitated adoptive immunotherapies of cancer23. Whether PRDM1 plays a hostile role in renal fibrosis needs further study. Then, we investigated the regulation of TGF-β on PRDM16. From the data shown above, PRDM16 was downregulated by TGF-β/Smad3 at the transcription level. Hariom Yadav et al showed that the expression of PRDM16 in fat tissue was increased after Smad3 knockout24. But the study didn't investigate the mechanism of TGF-β/Smad3 in regulating PRDM16 expression. We found that Smad3 activated by TGF-β decreased the transcription of PRDM16 by binding to the promotor directly. And through the proteomics analysis of pull-down products of PRDM16 promoter, we reported a novel cofactor of Smad3, H-Ras which played an important role in reducing PRDM16. We confirmed the interaction of H-Ras and p-Smad3 with Co-IP. The downstream of Smad3 is different when binds to different cooperators17,25. We found H-Ras from the pull-down complex of the PRDM16 promoter, indicating that the Smad3 specifically downregulated the expression of PRDM16 with the help of H-Ras possibly. The findings provide a novel precise regulation mechanism of PRDM16. Blocking the interaction between H-Ras and Smad3 can be a potential choice for attenuating renal fibrosis.

It is familiar that H-Ras is a member of GTPase which switches between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states26. The newly synthesized H-Ras locates in the cytoplasm27. However, the C-terminal hypervariable region (HVR) of H-Ras can be modified by lipids and anchors the G domain to the plasma membrane28. The H-Ras which resides on the plasma membrane can be recognized by the Ras binding domain of RAF and activates MAPK27. In this study, we found that H-Ras played as the co-activator of Smad3 in the nuclear. This subcellular localization is lack attention, but it exists. The evidence is as follows: 1. we extracted nuclear proteins to do DNA pull-down, and H-Ras can be detected in the immunocomplex; 2. we demonstrated that H-Ras interacted with p-Smad3, and p-Smad3 largely resided in nuclear29. 3. A widely used protein database, uniport shows the
subcellular locations of H-Ras are cell membrane, nucleus, cytoplasm, and Golgi apparatus. And we verified this phenomenon with an anti-H-Ras antibody from Proteintech (data was not shown).

From the analysis of RNA-sequence data, mitochondrial dysfunction was the most obvious change in the kidney after PRDM16 knockout. Mitochondrial dysfunction blocks fatty acid oxidation (FAO) which is the primary energy source for tubular epithelial cells, causing lipid accumulation, oxidative stress, partial epithelial to mesenchymal transition (partial EMT), and even cell death\textsuperscript{19,30}. Injured renal tubular epithelial cells secret multiple inflammatory factors and growth factors such as TGF-β, Wnt, and IL-1β to recruit immune cells and activate myofibroblasts, resulting in the progression of renal fibrosis \textsuperscript{31}. Consistently, Seale P. et al. found that adipocyte-specific knockout of PRDM16 caused brown adipocytes with small lipid droplets and rich mitochondria to lose their unique thermogenic genes, and converted them into white adipocytes containing large lipid droplets, low mitochondrial content \textsuperscript{32–34}. It was reported that TGF-β impaired mitochondrial function and fatty acid metabolism of tubular epithelial cells 30. PRDM16 overexpression blocked TGF-β induced mitochondrial dysfunction. The mechanism revealed by this study was that PRDM16 upregulated the protein and mRNA level of PGC-1α. Studies have shown that PGC-1α decreased greatly in CKD kidneys of mice and humans, and TGF-β contributed to this process\textsuperscript{5}. The downregulation of PGC-1α in renal tubular epithelial cells leads to mitochondrial dysfunction, increased ROS production, and intracellular lipid deposition\textsuperscript{35,36}. Overexpression of PGC-1α rescued the tubular mitochondrial dysfunction and fatty acid oxidation defect induced by Notch overexpression \textsuperscript{37}. The level of PGC-1α is positively correlated with glomerular filtration rate and negatively correlated with renal fibrosis\textsuperscript{38,39}. The possible mechanism underlying the regulation of PRDM16 on PGC-1α in adipocytes has been discussed partly. PRDM16 enhanced the activity of the PGC-1α promoter not by binding to the DNA directly. And PGC-1α protein which was a feedback transcription coactivator of its own gene was required for the regulation of PRDM16 on PGC-1α \textsuperscript{40–42}. Besides, PRDM16 inhibited the phosphorylation of Smad3 which transcriptionally downregulated the expression of PGC-1α\textsuperscript{24,43}. We showed that PRDM16 not only was downstream of TGF-β signaling but also inhibited the activation of Smad3 and ERK induced by TGF-β. It has been reported that PRDM16 repressed the transcription activity of TGF-β signaling and tumor cell proliferation by binding to p-Smad3 and suppressing the interaction of p-Smad3 to DNA \textsuperscript{43}. But how PRDM16 inhibits the activation of Smad3 and ERK is unclear. In the present study, PRDM16 also influenced the lipid deposition of tubular epithelial cells. One of the mechanisms is that the mitochondrial repair by PRDM16 facilitates fatty acid consumption by FAO \textsuperscript{11,44}. The other potential mechanism is that multiple FAO-related genes like \textit{CPT1A}, \textit{UCP-1}, and \textit{PPAR-α}, are activated by PRDM16 \textsuperscript{45–47}.

Finally, we demonstrated that the PRDM16 supplement protected kidneys from UUO and UIRI injury by preserving mitochondrial function. The previous studies indicate that the disappointing results of TGF-β inhibitors in clinical trials in large part relate to the inhibition of TGF-β in regulatory T cells which exacerbates autoimmune disease \textsuperscript{8}. Recently, it was reported that mitochondrial oxidative damage occurred in regulatory T cells of individuals with autoimmunity. Scavenging of mtROS attenuated
autoimmunity by preventing regulatory T cell death. PRDM16 shows a powerful effect on protecting mitochondrial function and improving oxidative phosphorylation. It can be suspected that PRDM16 supplement may restrict autoimmune disease by protecting regulatory T cells.

In summary, this study provides a novel target, PRDM16 for attenuating renal fibrosis. To apply the findings in the clinic, we will devote to overexpressing PRDM16 in vivo by designing mRNA drugs, screening agonists or mimic peptides in the future. And we illustrated that TGF-β decreased the transcription of PRDM16 in H-Ras/Smad3-dependent way. The compounds that block the interaction between H-Ras and Smad3 could also be potential options to treat CKD patients.

**Materials And Methods**

**Human renal biopsy samples**

Human renal biopsies were obtained from the Department of Nephrology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Fresh biopsies were dehydrated and paraffin embedded according to the routine procedures. Control samples were the healthy kidney tissues adjacent to kidney tumor. The extent of tubular atrophy and interstitial fibrosis which is < 25%, 25–50% and > 50% of the cortical area is defined as T0, T1, T2, respectively on the basis of the Oxford classification of IgA. The clinical characteristics and serum samples analysis are listed in Table S1. The investigation of human biopsy samples was approved by the Medical Ethics Committee of Union hospital, Tongji Medical College, Huazhong University of Science and Technology after the informed consent.

**Mice study**

Wild-type animal and transgenic mice were housed in a controlled environment (temperature 21 ± 1°C, humidity 50 ± 10%, and 12 h light/dark cycle) and were fed with free access to water and standard mouse chow diet (WQJX BIO-TECHNOLOGY). Cages with standard corncob bedding (WQJX BIO-TECHNOLOGY) were changed 2 times a week. For all of the in vivo experiments, littermate control mice were used. The number of mice used for all the experiment were mentioned in figure legends. All experiments for animal studies were approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology and obeyed the rules of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Generation of tubular epithelial cell-Specific Knockout Mice**

PRDM16^{floxt/floxf} mice (C57BL/6J;129) were generated by standard homologous recombination at Shanghai Southern Model Biotechnology Development Co., Ltd. (Shanghai, China). PRDM16 exon9 was flanked by loxP sequences. The PRDM16^{floxt/floxf} mice were cross mated with Ksp-cre mice. The breeding of transgenic mice was conducted by Shulaibao (Wuhan) Biotechnology Co., Ltd. PCR with the following primers: Forward CTGCTAAGGCCCTCTGGTAATAAC; Reverse AGCAGTGGGCAGGAGGGACAATG was used for mice genotyping with genomic DNA isolated from mouse tails.
UIRI and UUO model with transgenic mice

Male PRDM16$^{\text{floox/floox}}$ mice and Ksp-Cre/ PRDM16$^{\text{floox/floox}}$ mice aged 7–8 weeks were used. UUO and UIRI were performed as described in previous studies$^{29}$. To be specific, UUO model was that the left ureter was obstructed with 3 – 0 suture in the location paralleled to inferior pole of kidney. Mice were sacrificed in 7 days. Left kidney was collected to do further experiments. In UIRI model, pedicle of left kidney was clipped with vascular clamp, and mice were put on constant temperature metal bath at 38.5°C for 35 minutes. After taking down the clamps, the black ischemic kidney turned to red. Sham surgery was performed by exposure of kidney without ischemia and reperfusion. At 10 days after UIRI, right kidney was removed. Mice were sacrificed after 24 hours.

UIRI model with PRDM16 lentivirus

Male C57BL/6J;129 aged 7 weeks were purchased from Vital River Laboratory Animal Technology Co. Ltd. We first diluted lentivirus with ice-cold germfree PBS solution. Left kidney was exposed from the back of mice, and injected with lentivirus equivalently from the upper pole and inferior pole of kidney. 5×10$^6$ IU lentivirus in 100ul solution was injected to per kidney. 8mm insulin injectors were used to avoid bleeding. 7 days after lentivirus injection, ischemia reperfusion surgery as described above was conducted. Mice were put on constant temperature metal bath at 38.5°C for 40 minutes. The remaining steps were the same as the UIRI model of transgenic mice.

UUO model with PRDM16 plasmid

Hydrodynamics-based gene transfer method was used in this model$^{22}$. PRDM16 overexpression plasmid was obtained by inserting CDS sequence of PRDM16 into eukaryotic expressed GV141 plasmid. Mice were injected with 1mg/kg plasmid. PRDM16 overexpression plasmid was diluted with germfree PBS solution which was prewarmed to 37°C. Male C57BL/6J;129 aged 7 weeks were performed with UUO surgery. PRDM16 overexpression plasmid or blank plasmid was injected at the third and fifth day after UUO. Mice were sacrificed at day 7.

Cell Culture and Treatments

Human tubule epithelial cells (HK-2) were obtained from American Type Culture Collection (ATCC) and were cultured in cell incubator with 37°C, 5% carbon dioxide. DMEM/F12 (gibco) medium with 10% fetal bovine serum (FBS), and 100 U/mL penicillin plus 0.1 mg/mL streptomycin were used to culture HK-2 cells. For TGF-β treatment experiments, cells were seeded in 6 well plates, and cultured in serum-free medium with TGF-β (5ng/mL) after adherence. Cells were harvested in 24 hours for mitochondrial function detecting, and in 45 minutes for p-Smad3 and p-ERK detecting. In hypoxia and reperfusion experiments, hypoxia 24 hours and reperfusion 2 hours were used. SIS3(5 µmol, HY-13013, MCE) and ZLN005 (5 µmol HY-17538, MCE) were used.

Real time qRT-PCR
The mRNA of kidney tissue or cells was extracted by classical procedures with trizol and trichloromethane. 1µg mRNA was used to reverse transcription to cDNA with HiScript ® III RT SuperMix for qPCR (+ gDNA wiper) (R323-01, Vayzame, China). Real-time quantitative RT-PCR (qRT-PCR) was conducted on Step one plus system with the AceQ® qPCR SYBR® Green Master Mix (Q111, Vayzame, China). The reaction system(20µl) was constituted by 10µl SYBRmix, 0.4µl forward primers, 0.4µl reverse primers, 0.4µl ROX dye1, 3.8µl RNase free water and 5µl cDNA (diluted for 10 times). The specific primers for target genes in this study were listed in the table S2. The relative mRNA level of genes was calculated with 2-ΔΔCT, and normalized with the housekeeping gene β-actin.

**Western Blot Analyses**

Western blot was performed as described previously29. In brief, tissues or cultured cells were lysed in RIPA buffer (G2002, Servicebio, China) containing 1x protease inhibitor cocktail (G2006, Servicebio, China), 1xPMSF (ST506, Beyotime Biotechnology, China) for at least 10mins on ice and centrifugated at 4°C for 15mins 12000 rpm. The supernatant of centrifugation was collected, and the concentration of protein was calculated with BCA Elisa KIT (AR0133, G-Clone, China). Proteins were separated by 8% or 10% SDS-PAGE and transferred onto 0.45µm PVDF membranes. Proteins were incubated with primary antibodies at 4 °C overnight and secondary antibodies at room temperature for 1h. Antibodies used in this study are summarized in Table S3. Bands were visualized by enhanced chemiluminescence (Applygen, Beijing, China). The housekeeping protein α-tubulin, β-actin and GAPDH were used as loading control.

**Immunofluorescence staining**

The 2µm frozen renal sections or cultured cells on coverslips were fixed with 4% paraformaldehyde solution for 15mins, then treated with 0.2% triton X-100 for 15mins. After 1h blocking by 10% donkey serum, sections were incubated with primary antibodies at 4°C overnight, secondary antibodies at 37°C 1h. The nuclei were stained by DAPI (G1012, Servicebio, China). The figures of stained samples were collected on laser scanning confocal microscope (Dragonfly/CR-DFLY-201-40, ANDOR) or fluorescence microscope (DMi8, Leica).

**Immunohistochemical Staining**

The 3µm paraffin sections were immersed in xylol overnight. Sections were dewaxed with xylol, anhydrous ethanol, 95% ethanol, and 75% ethanol. 3% Hydrogen peroxide methanol was used to consume endogenous peroxidase. The citrate antigen unmasking solution was used in antigen retrieval with microwave methods. After 1h blocking by 10% donkey serum, sections were incubated with primary antibodies at 4°C overnight, secondary antibodies at 37°C 1h. The nuclei were stained by Hematoxylin. Stained samples were viewed under a bright field microscope (Ni-E, Nikon) equipped with a digital camera. Immunohistochemistry analysis were performed as described previously.

**ATP detecting**
The measurement of ATP level was conducted by ATP Assay Kit (S0026, Beyotime Biotechnology, China). Tissue samples or cultured cells were lysed at 4°C for 15 mins and centrifuged at 4°C for 15 mins 12000 rpm. The supernatant of centrifugation was collected and luminescence were detected by ATP Assay Kit on microplate reader (PerkinElmer Multimode). The level of sample ATP were standardized with ATP standard.

**Oil Red O Staining**

Cells cultured on coverslips were washed using PBS, fixed using 4% PFA solution and stained for 10 min with Oil-red O working solution (Sigma-Aldrich 00625-25G) to visualize lipid droplets. Cell nuclei were stained by Hematoxylin (G-clone, Beijing, China). Animal frozen renal tissues were sectioned into 2µm, fixed by 4% PFA solution for 30 mins, and stained for 15 min with Oil-red O working solution. Then cell nuclei were stained by Hematoxylin.

**Masson-trichrome staining**

The paraffin sections in 3µm were used to dewax and rehydration. Then sections were soaked in Bouin solution at 65°C for 3 h. After washing, sections were stained by hematoxylin, ponceau staining solution and aniline blue staining solution. And sections were differentiated with 1% hydrochloric acid alcohol and then treated with TO transparent agent.

**Hematoxylin-eosin (HE) staining**

Steps of dewaxing and rehydration were same with immunohistochemical staining. Sections were stained by Hematoxylin for 10 minutes and Eosin for 30 s. And sections were differentiated with 1% hydrochloric acid alcohol and then treated with TO transparent agent.

**Sirius Red staining**

The paraffin sections in 6µm were used. Step of dewaxing and rehydration were same with IHC. Sections were stained by Sirius Red staining solution (G-clone, Beijing, China) at 37 °C for 1 h. Then sections were washed by purified water.

**Mitotracker Staining**

Cells cultured on coverslip were washed using PBS, then incubated with mitotracker (Invitrogen M22425, USA) at 37°C 30 mins. The coverslips were fixed using 4% PFA solution and stained by Actin-Tracker Green (C1033, Beyotime Biotechnology, China). Cell nuclei were stained by DAPI (G1012, Servicebio, China). Animal tissue were frozen sectioned into 2µm, incubated with mitotracker (Invitrogen M22425, USA) at 37°C 30 mins, then fixed using 4% PFA solution. The nuclei were stained by DAPI (G1012, Servicebio, China).

**Bodipy Staining**

Cells cultured on coverslip were washed using PBS, incubated with Bodipy working solution (M9850, AbMole, USA) at 37°C for 30 mins. Then coverslips were fixed using 4% PFA solution. Cell nuclei were
stained by DAPI (G1012, Servicebio, China). Animal tissue were frozen sectioned into 2µm, incubated with Bodipy working solution (M9850, AbMole, USA) at 37°C for 60 mins. then fixed using 4% PFA solution. The nuclei were stained by DAPI (G1012, Servicebio, China).

**Biochemical Analysis of Serum Samples**

The clinical parameters of serum such as AST, ALT, BUN and glucose were measured by automatic biochemical analyzer. Serum creatine was measured by creatine assay kit (DICT-500, bioassay systems, USA).

**Co-Immunoprecipitation (Co-IP)**

The Co-Immunoprecipitation (Co-IP) was performed by CO-IP Kit (P2197, Beyotime Biotechnology, China). In brief, HK-2 cells which were treated with TGF-β for 45 minutes were lysed in IP lysis with 1× protease inhibitor cocktail and 1×PMSF for at least 10 minutes on ice and centrifugated at 12000 rpm 4°C for 15mins. 20µl supernatant was collected as input. The rest supernatant was incubated with arogase A + G and 1µg antibody (primary antibody or Rabbit IgG) at 4°C. The mixture was diluted with pre-cold PBS to 500µl, and rotated overnight. After washing with pre-cold PBS solution, the pull-down proteins were boiled with loading buffer at 100°C metal bath for later immuno-blotting.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed by ChIP Kit (P2078, Beyotime Biotechnology, China). In brief, the culture medium was added with 1% methanal to fix cells, and added 1× Glycine Solution, then washed by 1×PBS with 1% PMSF according to the instruction manual. Cells were collected and resuspended by SDS Lysis buffer. And DNA was crushed by ultrasonic equipment. The DNA fragments were diluted by ChIP dilution buffer. 20µl of the sample was collected as input. The rest of the samples were incubated with Protein A + G Agarose/Salmon Sperm DNA and 3µg primary antibody at 4°C overnight at rotary table. Sample then were washed and purified to conduct PCR amplification and DNA gel electrophoresis.

**DNA-pull down**

The HK-2 cells were treated with 5ng/mL TGF-β for 24 hours, and 5×10^7 cells were collected and extracted their nucleus proteins with Kit (Thermo fisher, 78833). To obtain biotin labeled DNA probe of PRDM16 promoter, PRDM16-luc plasmid, forward primer: bio-TTCTCTGCCCCAACCCCTG, reverse primer: GGTGTCGCTCGCGGAATC, PrimeSTAR Max Premix (2×) (Takara, R045B) were used to PCR amplification. And DNA was recycled after gel electrophoresis with agarose gel DNA Recovery Kit (Guangzhou IGE biotechnology ltd, K110-S, China). Then the nucleus proteins were incubated with biotin labeled DNA probe of PRDM16 promoter or DNA probe without label for 4°C overnight. Next day, streptavidin magnetic beads (Thermo fisher, 21344, UAS) were added to the tubules to pull down the DNA-protein complex. After washing and elution, the final pull-down proteins were measured by LC/MS-MS. DNA Probe synthesis, pull-down and LC/MS-MS were performed at Fitgene Biotech Co., LTD.

**Lentivirus-Mediated Gene Expression**
The HK-2 cell were cultured in 6 wells culture plates at 20–30% density. Cells were treated with co-transfection reagent A and P, and lentivirus. The multiplicity of infection (MOI) of HK-2 was 10. 12h after transfection, medium was changed. 24h after transfection, GFP florescence were observed. 72h after transfection, 2µg/ml puromycin was added to medium to kill the non-transfected cells.

**siRNA-mediated Knockdown**

HK-2 cells were cultured and seeded on 6 well plate with 50–60% density. Cells were changed to optimem culture medium. 5µl siRNA was transfected to each well with 5µl lipo2000 lipofectamine (Invitrogen, Carlsbad, CA). Cells were changed to DMEM/F12 medium with 10%FBS for 24 hours.

**RNA-sequencing Analysis**

Total RNA was extracted from HK-2 cells which were treated with TGF-β for 24 hours, and total RNA was extracted from the kidney cortex of PRDM16<sup>floxflox</sup> and Ksp-cre/PRDM16<sup>floxflox</sup> mice which suffered from UIRI surgery, and were subjected to RNA-seq analysis performed by MGI platform. Briefly, RNA quality was checked by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The mRNA purified by Oligo(dT)-attached magnetic beads was fragmented into small pieces with fragment buffer at appropriate temperature. Then First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis and purified using AMPure XP Beads. The cDNA library was constructed through repair, PCR amplification, and purification. Qubit 2.0 and Agilent 2100 bioanalyzer were used for quantification. Sequencing was performed by BGI Hong Kong on the MGI DNBseq T7 platformas 150 bp paired-end reads, the data of per sample was 6G. Raw reads from RNA-seq libraries are filtered to remove reads containing adapters or reads of low quality. After filtering, statistics analysis was performed using R software. The library construction, sequencing, and analysis were performed at Wuhan Generead Biotechnology Co. Ltd (Wuhan, China).

**Transmission Electron Microscopy**

To observe the structure of mitochondria, HK-2 cells and kidney tissues were collected and fixed with 2.5% glutaraldehyde at 4°C. Then, the samples were washed 15 minutes with 0.1 mol/L PBS for 6 times, and fixed with 1% osmium tetroxide for 1 hour at room temperature. After fixation, samples were dehydrated with 3% acetone 15minutes, 5% acetone 15minutes, 70% acetone 15minutes, 90% acetone 15minutes, 100% acetone 10minutes for 4 times in series. And next, ethoxyline resin was used to embed samples for 12 hours at 37°C. Finally, we can observe the samples by transmission electron microscope after section. Sample handling and detection were conducted by the electron microscopic lab of Department of nephrology, Union hospital, Huazhong science and technology university.

**Quantification and statistical analysis**

All data are expressed as mean ± SEM. P < 0.05 was considered significant. Two-tailed Student’s unpaired t test analysis was used to compare between two groups. One-way ANOVA followed by Tukey’s post-test
was used to compare more than three groups. Spearman correlation analysis was used to assess the relationship between the ratio of PRDM16 positive area and other variables.

**Declarations**

All authors declared no competing interests.

**Author contributions**

Q.Y. and C.Z. designed the study; Q.Y, B.T, H.S collected and analyzed the clinical data; Q.Y, B.T, W.C, YR.X, YJ.X, YT.Z performed animal models; Q.Y, B.T, YR.X performed in vitro experiments; Q.Y, B.T prepared figures and tables; Q.Y, B.T wrote the paper; C.Z. revised and approved the final version of manuscript.

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**References**


**Figures**
PRDM16 was decreased in the injured kidneys of mice and humans.

(a) Fold change of PRDM-family in HK-2 cells treated with TGF-β for 24 hours (n= 3). (b) The expression of PRDM16 in normal control and CKD patients from the nephroseq database. (c-d) Representative immunohistochemical staining images of PRDM16 (c) and the quantification of PRDM16 positive stained area (d) in the renal biopsy sample of IgA nephropathy patients (T0-T2) (n=4). Control samples were derived from the healthy kidney poles of individuals who underwent tumor nephrectomies (n=3). Scale bar: 50μm. (e) Correlation between the serum urea nitrogen level and the quantification of PRDM16 positive stained area in all subjects (n=14). (f-g) Representative Western blotting (f) (n = 4 blots in total) and quantification of PRDM16 (g) of sham and UUO kidneys. (h) Relative mRNA level of *PRDM16* in UUO mice (n=6). (i-j) Representative Western blotting (i) (n = 4 blots in total) and quantification of PRDM16 (j) in the kidney from UIRI mice. (k) Relative mRNA level of *PRDM16* in sham and UIRI kidneys (n=6). (l) Representative immunohistochemical staining images of PRDM16 in the kidney of sham and UUO mice. Scale bar: 20μm. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Two-tailed Student’s unpaired t test analysis (b, d, g, h, j, k), Spearman's correlations (e).
Figure 2

**TGF-β transcriptionally downregulated PRDM16 in an H-Ras/p-Smad3 dependent way.**

(a-b) Representative Western blotting (a) (n=4 or 5 blots in total) and quantification of PRDM16 (b) in HK-2 cells treated with 5ng/ml TGF-β for 24 hours. (c) Relative mRNA level of PRDM16 (n=3). (d-e) The protein lysate of HK-2 cells which were treated with 5ng/ml TGF-β, and SIS3 (5μm/L) for 24 hours was...
used. Representative Western blotting (n= 3 blots in total) and quantification of PRDM16 were shown. (f) Relative mRNA level of PRDM16 (n=3). (g) The predictive motif of Smad3 DNA-binding domain from jaspar.genereg.net and the latent binding site of Smad3 at PRDM16 promoter. Primers 1 and 2 were designed targeting to -1884 to -1875; Primer3 was designed targeting to -33 to -24. (h) Representative images of ChIP-PCR in HK-2 cells treated with TGF-β 5ng/ml or vehicle for 45 minutes. (i) Schematic diagram of DNA pull-down. (j) The Venn diagram showed that differential proteins in the DNA pull-down complex shared 5 proteins with the GO set of Wound healing (GO:0042060). (k) The mass spectrum of H-Ras. (l) Relative mRNA level of H-Ras in HK-2 cells (n=3) transfected with H-Ras siRNA (H-Ras-si) or Scramble. (m-n) Representative Western blotting (m) (n = 3 blots in total) and quantification of PRDM16, PGC-1α and fibronectin (n) in HK-2 cells treated with 5ng/ml TGF-β, and H-Ras siRNA transfection for 24 hours. (o) Protein-Protein interaction network of H-Ras and Smad3 by STRING database. (p) Co-immunoprecipitation of H-Ras and p-Smad3 in HK-2 cells treated with 5ng/ml TGF-β for 45 minutes. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; Two-tailed Student’s unpaired t test analysis (b, c, l), One-way ANOVA followed by Tukey’s post-test (e, f, n).
Figure 3

Tubular-specific knockout PRDM16 aggravated ischemia-reperfusion-induced renal interstitial fibrosis by promoting mitochondrial dysfunction.

(a-b) The measurement of serum creatine level (a) and blood urea nitrogen (BUN) level (b) of \(PRDM16^{\text{floox/floox}}(PR^{+/+})\) and \(Ksp-cre/PRDM16^{\text{floox/floox}}(PR^{+/f})\) mice suffered from UIRI or sham surgery (n=6).
(c) Volcano plot of RNA-sequencing. (d) Heat map of RNA-sequencing. (e) GSEA analysis of RNA-sequencing. (f) KEGG analysis of RNA-sequencing. (g) Representative images of mitotracker and Borondipyrromethene (Bodipy) staining. Scale bar: 25μm. (h-j) Representative Western blotting (h) (n = 6 blots in total) and quantification of PGC-1α (i) and α-SMA (j) with kidney lysate of PR+/+ and PR+/− mice. (k-l) Representative images of Masson-trichrome staining (MTS) (k) and quantification of fibrotic area (l) of each group (n=6). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; One-way ANOVA followed by Tukey’s post-test (a, b, i, j, l).
Figure 4

Tubular-specific knockout PRDM16 promoted tubular mitochondrial injury and interstitial fibrosis of UUO mice.

(a) The representative kidney cross section from each group. (b) Relative mRNA level of Acox2, Ppargc1a, and Tfam in renal cortex from PR^{+/+} and PR^{+/fl} mice (n=6). (c) Representative images of Transmission Electron Microscope (TEM) showed the structure and number of mitochondria in tubular epithelial cells. Scar bar: 30μm. (d-e) Representative Western blotting (d) (n = 6 blots in total) and quantification (e) of PGC-1α, α-SMA and Fibronectin (n=6). (f-i) Relative mRNA levels of fibronectin, Col1a1, Col3a, and Vimentin genes (n=6). (j-k) Representative images of Masson-trichrome staining (j) and quantification of fibrotic area (k) of each group (n=6). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; One-way ANOVA followed by Tukey’s post-test (b, e-i, k).
**Figure 5**

**PRDM16 inhibited TGF-β induced tubular mitochondrial dysfunction and TGF-β signaling.**

(a) Relative mRNA level of *CPT1A*, *TFAM*, and *ACOX2* in control lentivirus (NC-LV) transfected HK-2 cell lines and PRDM16 overexpression lentivirus (PR-LV) transfected HK-2 cell lines treated with 5ng/ml transforming growth factor-β (TGF-β) for 24 hours (n=3).  
(b-d) Representative Western blotting (b) (n = 3...
blots in total) and quantification of PPAR-γ (c) and CPT1A (d) in stably transfected HK-2 cells after TGF-β treatment for 24 hours. (e) Representative images of mitotracker staining, Oil-red O staining and Transmission Electron Microscope (TEM). In the first line of the Fig.s, red was mitotracker staining, green was phalloidin staining, blue was DAPI. Scale bar: 10μm. (f) The measurement of adenosine triphosphate (ATP) level in stably transfected HK-2 cells treated with TGF-β (n=3). (g-i) Stably transfected HK-2 cells were harvested after 5ng/ml TGF-β treatment for 45 minutes. Representative Western blotting (g) (n = 3 blots in total) and quantification of phospho-ERK (p-ERK) (h) and phospho-Smad3 (p-Smad3) (i). (j) Representative images of immunofluorescence of p-Smad3 in cells treated with 5ng/ml TGF-β for 45 minutes. Scale Bar: 10μm. White Arrow: positive staining. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; one-way ANOVA followed by Tukey's post-test (a, c, d, f, h, i).
Figure 6

PRDM16 rescued tubular mitochondrial function via upregulating PGC-1α.

(a) Representative immunohistochemical staining images of co-staining of PRDM16 and PGC-1α by serial renal biopsy section of CKD patients. Scale bar: 20μm. The black arrow pointed to positive co-staining tubules. (b) Correlation between the quantification of positive stained area of PRDM16 and PGC-
1α (n=15). (c-f) HK-2 cells were transfected with PRDM16 siRNA or Scramble for 24 hours. Representative Western blotting (c) (n = 3 blots in total) and quantification of PRDM16 (d), PGC-1α (e), and PPAR-γ (f). (g-h) Representative Western blotting (n = 3 blots in total) and quantification of PGC-1α in stably transfected HK-2 cells after TGF-β treatment for 24 hours. (i) Relative mRNA level of PPARGC1A in stably transfected HK-2 cells after TGF-β treatment for 24 hours. (n = 3) (j-l) Stably transfected HK-2 cells were treated with hypoxia for 24 hours and reperfusion for 2 hours. Representative Western blotting (j) (n = 3 blots in total) and quantification of PGC-1α (k) and PPAR-γ (l). (m-o) HK-2 cells were treated with TGF-β, PRDM16 siRNA (PR-siRNA) or Scramble, and ZLN005 for 24 hours according to the grouping. Representative Western blotting (m) and quantification of PGC-1α(n) and fibronectin (o) were shown. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; Spearman’s correlations (b), Two-tailed Student's unpaired t-test analysis (d-f), one-way ANOVA followed by Tukey’s post-test (h, i, k, l, n, o).
Figure 7

Renal injection with PRDM16 overexpressing lentivirus attenuated mitochondrial dysfunction and interstitial fibrosis in the UIRI model.

(a) schematic diagram of UIRI model with lentivirus injection: left kidney was injected with $5 \times 10^6$ IU control lentivirus (NC-LV) or PRDM16 overexpression lentivirus (PR-LV). After 7 days, unilateral ischemia...
reperfusion surgery was performed to the same kidney. And the contralateral kidney was removed in 10 days after UIRI surgery. Mice were sacrificed at day 11. (b-c) Level of serum creatine and BUN in each group (n=6 or 8). (d) Representative immunohistochemical staining images of PRDM16. Scale bar: 20μm. (e) Ratio of PRDM16 positive staining area to total area (n=6 or 8). (f) Relative mRNA level of PRDM16 in renal cortex of each group (n=6 or 8). (g) Representative immunohistochemical staining images of PGC-1α. Scale bar: 20μm. (h) Ratio of PGC-1α positive staining area to total area (n=6 or 8). (i) Relative mRNA level of Ppargc1a in renal cortex of each group (n=6 or 8). (j-m) Representative Western blotting (j) (n = 6 or 8 blots in total) and quantification of PGC-1α (k), α-SMA (l) and Fibronectin (m) with kidney pole cortex lysate. (n) Representative figures of mitotracker staining. Scale bar: 10μm. (o) Representative figures of Oil-red O staining. Scale bar: 20μm. (p) Representative images of immunohistochemical staining of α-SMA and Masson-trichrome staining. Scale bar: 20μm. (q) Quantifications of α-SMA positive staining area (n=6 or 8). (r) Quantifications of fibrotic area (n=6 or 8). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; One-way ANOVA followed by Tukey’s post-test (b, c, e, f, h, i, k-m, q, r).
Figure 8

PRDM16 overexpression also ameliorated mitochondrial dysfunction and interstitial fibrosis of UUO mice.

(a) Representative immunohistochemical staining images of PRDM16. Scale bar: 50μm. (b-c) PRDM16 was overexpressed in UUO kidneys through the hydrodynamic-based gene delivery method. Representative Western blotting (b) (n = 6 blots in total) and quantification of PRDM16 (c). (d) Relative
mRNA level of PRDM16 (n=6). (e) Representative immunohistochemical staining images of PGC-1α. Scale bar: 50μm. (f-g) Representative Western blotting (f) (n = 6 blots in total) and quantification of PGC-1α (g). (h) Relative mRNA level of Ppargc1a (n=6). (i) Measurement of renal ATP level in UUO model (n=6). (j) Representative images of mitotracker staining. Scale bar: 50μm. (k) Representative images of Oil-red O staining. Scale bar: 50μm. (l-n) Representative images (l), and quantification of Sirius Red Staining and Masson-trichrome staining (m) and immunohistochemical staining of fibronectin (n) (n=6). Scale bar: 50μm. (o-p) Relative mRNA level of fibronectin and Col1a1 in the kidney of mice (n=6). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; †p < 0.05, ††p < 0.01, †††p < 0.001; One-way ANOVA followed by Tukey’s post-test (c-d, g-i, m-p).

Figure 9

Schematic diagram shows that PRDM16 is a novel downstream of TGF-β/Smad3 signaling that regulates renal fibrosis. In fibrotic kidney, phosphorylated Smad3 triggered by TGF-β interacts with H-Ras, and binds to the promoter of PRDM16 to downregulate its transcription. PRDM16 deficiency results in mitochondrial injury and lipid deposition by decreasing PGC-1α, leading to the progression of renal fibrosis. PRDM16 supplement rescues renal fibrosis of UUO and UIRI mice.

Supplementary Files

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