

Original Research

# TRIM6 Promotes ROS-Mediated Inflammasome Activation and Pyroptosis in Renal Tubular Epithelial Cells via Ubiquitination and Degradation of GPX3 Protein

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Academic Editor: Viviana di Giacomo

Submitted: 17 August 2023 Revised: 8 September 2023 Accepted: 9 January 2024 Published: 5 February 2024

## Abstract

**Background:** Pyroptosis is a critical form of cell death during the development of chronic kidney disease (CKD). Tripartite motif 6 (TRIM6) is an E3-ubiquitin ligase that participates in the progression renal fibrosis (RF). The aim of this study was to investigate the roles of TRIM6 and Glutathione peroxidase 3 (GPX3) in oxidative stress-induced inflammasome activation and pyroptosis in Ang-II treated renal tubular epithelial cells. **Methods:** To study its role in RF, TRIM6 expression was either reduced or increased in human kidney-2 (HK2) cells using lentivirus, and Ang-II, NAC and BMS-986299 were served as reactive oxygen species (ROS) inducer, ROS scavenger and NLRP3 agonist respectively. Pyroptosis and mitochondrial ROS were measured by flow cytometry. The levels of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) were determined using commercial kits, while the levels of IL-1 $\beta$ , IL-18, IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were determined by Enzyme-Linked Immunosorbent Assay (ELISA). Co-immunoprecipitation (Co-IP) assay was used to evaluate the interaction between TRIM6 and GPX3. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot were used to measure mRNA and protein expression, respectively. **Results:** Treatment with Angiotensin II (Ang II) increased the protein and mRNA levels of TRIM6 in HK2 cells. Ang II also increased mitochondrial ROS production and the malondialdehyde (MDA) level, but decreased the levels of GSH and SOD. In addition, Ang II enhanced HK2 cell pyroptosis, increased the levels of IL-1 $\beta$ , IL-18, IL-6, and TNF- $\alpha$ , and promoted the expression of active IL-1 $\beta$ , NLRP3, caspase-1, and GSDMD-N proteins. These effects were reversed by knockdown of TRIM6 and by treatment with N-acetyl-L-cysteine (NAC), a ROS scavenger. BMS-986299, an NLRP3 agonist treatment, did not affect ROS production in HK2 cells exposed to Ang II combined with NAC, but cell pyroptosis and inflammation were aggravated. Moreover, the overexpression of TRIM6 in HK2 cells resulted in similar effects to Ang II. NAC and GPX3 overexpression in HK2 cells could reverse ROS production, inflammation, and pyroptosis induced by TRIM6 overexpression. TRIM6 overexpression decreased the GPX3 protein level by promoting its ubiquitination, without affecting the GPX3 mRNA level. Thus, TRIM6 facilitates GPX3 ubiquitination, contributing to increased ROS levels and pyroptosis in HK2 cells. **Conclusions:** TRIM6 increases oxidative stress and promotes the pyroptosis of HK2 cells by regulating GPX3 ubiquitination. These findings could contribute to the development of novel drugs for the treatment of RF.

**Keywords:** renal fibrosis; oxidative stress; pyroptosis; ubiquitination; tripartite motif containing proteins

## 1. Introduction

Chronic kidney disease (CKD) is a progressive clinical condition characterized by renal structural change and renal function loss. CKD develops under the influence of various risk factors, such as obesity, diabetes and infection [1]. The World Health Organization has listed CKD as a major public health problem worldwide, with a global prevalence of 13.4% [2]. China has a large population with CKD and an overall prevalence of 11.6% for this disease [3]. Effective therapeutic options for CKD are currently limited [4]. Therefore, elucidation of the underlying mechanism of CKD may help with disease prevention, or with the delay of disease progression.

A stable rate of production and degradation of reactive oxygen species (ROS) in the human body is required for the removal of harmful substances. However, failure to detoxify these reactive products leads to cell and tissue accumulation of ROS, thereby harming organ function [5]. Oxidative stress acts as a pivotal factor in the progression of renal fibrosis (RF). The resulting glomerular lesions and renal ischemia lead to inflammation and endothelial dysfunction [6]. Patients with advanced-stage CKD were found to have increased ROS levels. This plays a crucial role in the development of RF by initiating an inflammatory chain reaction involving the recruitment of infiltrating macrophages and the secretion of pro-fibrogenic and pro-inflammatory cytokines [7]. It has also been shown that renal dysfunction



damages the antioxidant system, thereby making patients highly susceptible to oxidant stress [8]. However, the exact role of ROS in CKD requires further investigation.

Our previous published article revealed that Angiotensin (Ang) II is a powerful inducer that can not only induce production of epithelial mesenchymal transition (EMT), but also promote the production of ROS that further mediates the activation of inflammasomes, a crucial process tightly associated with different renal diseases [9]. N-acetylcysteine (NAC) is an amino acid with multiple biological activities that can exert antioxidant and anti-inflammatory effects through inhibiting the production and release of ROS as well as the release of inflammatory cytokines, which plays an important role in reducing kidney inflammation and delaying the progression of renal fibrosis.

Inflammasomes are cytoplasmic multiprotein complexes that can be triggered by endogenous and exogenous stimuli. The mechanism of inflammasome activation involves formation of protein complexes that activate caspase 1, which then proteolytically activates pro-inflammatory cytokines such as IL-1 $\beta$  and IL-18 [10]. A certain degree of inflammasome activation is beneficial for the host by eliminating foreign pathogens and repairing damaged tissues, but hyperactivation of inflammasomes can contribute to various diseases [11]. For example, inflammasome activation has been shown to participate in post-ischemic inflammation after stroke [12]. Extensive evidence suggests that Nucleotide-binding domain, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3)-mediated inflammasome activation is a vital contributor to diabetes-related vascular dysfunction and to pro-inflammatory phenotypes [13]. A previous study reported that ROS act as a regulator and trigger of the NLRP3 inflammasome, which then causes Caspase-1-dependent cell pyroptosis [14]. Inflammasomes also play an important role in CKD. The inhibition of inflammasome activation has been shown to protect against dysregulated mitochondrial function and RF [15]. Despite numerous studies on the role of the inflammasome in CKD, the underlying mechanism remains to be elucidated.

The Tripartite motif (TRIM) family of proteins has three conserved domains. From the N-terminal to the C terminal, these are a RING finger, one or two B-boxes, and a coiled-coil (CC) domain [9]. Major importance has been attached to the function of TRIM proteins due to their E3 ubiquitin ligase activity associated with the RING finger structure [16]. Pyroptosis is a type of programmed cell death, also referred to as cellular inflammatory necrosis, with features of cell swelling and cell membrane rupture that eventually lead to the massive leakage of cellular contents. Studies have revealed that TRIM proteins are closely involved in a variety of biological and pathobiological processes, including pyroptosis [17,18]. A previous study reported that enhanced expression of Tripartite motif 6 (TRIM6) was observed in RF, and that TRIM6 activates mTORC1 by

ubiquitinating TSC1-TSC2, thereby promoting endoplasmic reticulum stress, EMT, and RF [9]. However, the mechanism that involves TRIM6 in CKD is not well understood. Ang II has been reported to be involved in various biological processes, including the promotion of EMT and RF [19], NLRP3 inflammasome activation [20], and the induction of ROS [9]. Ang-II is one of the major contributors to the progression of renal fibrosis, inflammation, glomerular injury, and chronic kidney disease [19]. The aim of this study was to further elucidate the functional role of TRIM6 in RF by using Ang II as a tool. The results should lead to a better understanding of the underlying molecular mechanism of RF.

## 2. Material and Methods

### 2.1 Cell Culture

Human kidney-2 (HK2) cells free of mycoplasma contamination were obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai Biology Institute (#SCSP-511, Shanghai, China). HK2 cells were cultured in RPMI 1640 medium (#11875093, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (#16140071, Gibco), penicillin (100 IU/mL), and streptomycin (100  $\mu$ g/mL) in an incubator (37 °C, 95% humidity, 5% CO<sub>2</sub>). Cells in the short interfering RNA negative control (shNC) group were cultured with negative control shRNA. All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells in the Ang II group were cultured with 1  $\mu$ M Ang II. Cells in the TRIM6 knockdown (shTRIM6) group were transfected with shTRIM6 lentivirus. Cells in the NLRP3 agonist (BMS-986299) group were cultured with 1  $\mu$ M BMS-986299 (#2242952-69-6, MCE, Shanghai, China). Cells in the TRIM6 overexpression (oeTRIM6) group were transfected with oeTRIM6 lentivirus. Cells in the N-acetylcysteine (NAC) group were cultured with 100  $\mu$ M NAC (#616-91-1, Billerica, MA, USA). Cells in the Glutathione peroxidase 3 (GPX3) overexpression (oeGPX3) group were transfected with oeGPX3 lentivirus. The various evaluations were performed after 24 hours of cell cultivation.

### 2.2 Lentivirus Preparation

Full length human GPX3 was cloned into pLVX-puro (Clontech, Palo Alto, CA, USA). The lentivirus expressing short hairpin RNA (shRNA) oligos targeting TRIM6 (shTRIM6#1, #2), TRIM6 (oeTRIM6) or GPX3 (oeGPX3) was produced in 293T cells, along with the packaging plasmids, psPAX2 and pMD2.G as previously described (2  $\mu$ g/mL) [9]. The viral supernatant was harvested after 48 h ( $1 \times 10^8$  transducing units (TU)/mL). Then, HK2 cells were seeded into 24-well plates and transfected with lentivirus vectors.

### 2.3 Determination of ROS

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a ROS fluorescence probe. DCFH-DA by itself is not fluorescent and can pass freely through the living cell membrane. After entering the cell, DCFH-DA is hydrolyzed by esterases to generate DCFH, which cannot penetrate the cell membrane. Intracellular ROS can oxidize non-fluorescent DCFH to fluorescent DCF. The intracellular ROS level can be determined by measuring the fluorescence of DCF. At the end of cell interventions, samples were collected and washed with ice-cold PBS. A ROS detection kit (#S0033S, Beyotime, Shanghai, China) was used to treat cells according to the instructions, and flow cytometry was used to evaluate ROS level in samples. Each assay was repeated in triplicate.

### 2.4 Evaluation of Pyroptosis-Related Protein

At the end of cells intervention, samples were collected and washed with ice-cold PBS. Then, cells were incubated with a caspase-1 detection probe (FAM-FLICAR Caspase 1 Assay Kit, #ICT-97, ImmunoChemistry Technologies, Davis, CA, USA) for 1 h at 25 °C in the dark. To remove non-combined caspase-1, the cells were washed with PBS for three times and incubated with propidium iodide solution (Propidium (PI), #P3566; Invitrogen, Carlsbad, CA, USA) at 3 μM for 15 min in the dark. Thereafter, the cells were analyzed using flow cytometry (BD FACSCalibur, Franklin Lake, NJ, USA). The pyroptosis cells were determined by forward scatter/side scatter (FSC/SSC). The cells in Upper Right quadrant (Q1 UR) and Upper left quadrant (Q1-UL) were PI positive cells, and the cells Q1-UR and Q1-LR are caspase-1 positive cells. Activate caspase-1 and PI double positive cells were defined as pyroptotic cells. Each assay was repeated in triplicate.

### 2.5 MDA, GSH and SOD Assays

The levels of malondialdehyde (MDA) and glutathione (GSH) were measured using the thiobarbituric acid method and the DTNB (5,5'-dithiobis-2-nitrobenoic acid) method, respectively. The superoxide dismutase (SOD) inhibition rate was measured using the WST-1 method (The WST-1 method is a commonly used method for measuring cell proliferation based on water-soluble tetrazolium salt and the principle of dehydrogenase metabolism). The commercial kits for these assays were #A003-1, #A006-2, and #A001-3, respectively, from the Jiancheng bioengineering institute, Nanjing, China. The manufacturer's standard procedure was followed for all three assays.

### 2.6 Evaluation of IL-1β, IL-18, IL-6, and TNF-α Levels

The supernatant containing IL-1β, IL-18, IL-6, and tumor necrosis factor-α (TNF-α) was harvested from the culture medium. The expression levels for these factors were evaluated according to recommended protocols using the enzyme-linked immunosorbent assay (ELISA) kits

#H002-1, #H015-1, #H007-1, and #H052-1, respectively, from the Jiancheng bioengineering institute. Each assay was repeated in triplicate.

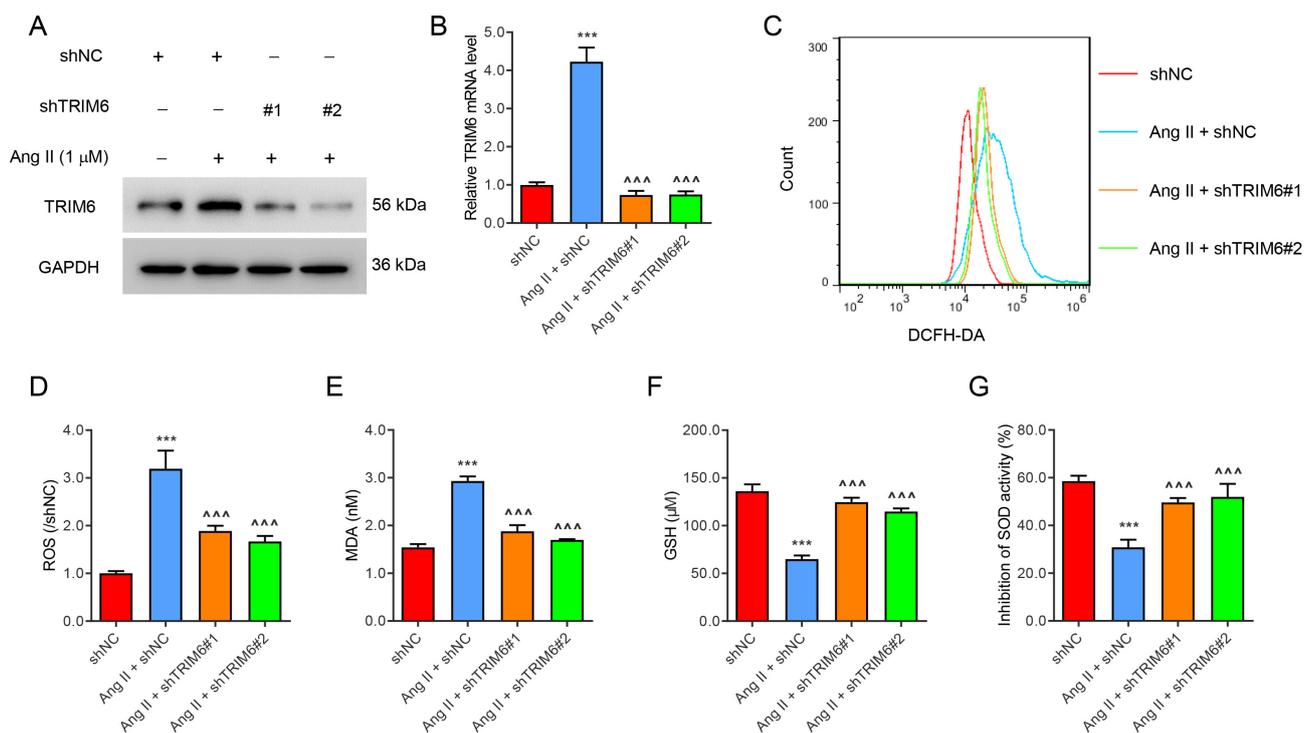
### 2.7 Evaluation of mRNA Expression

The procedure for quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was described in our previous report [9]. Total RNA was extracted from HK-2 cells with Trizol reagent (Invitrogen, Waltham, MA, USA). Reverse transcription was performed using a first-strand cDNA reverse transcription kit (Takara, Dalian, Liaoning, China). Quantitative RT-PCR was employed to measure the mRNA levels of specific genes with the SYBR®Green kit (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA level of genes was measured with the  $2^{-\Delta\Delta CT}$  method. All data represent the average of three replicates. The primer sequences are: TRIM6, 5' CTTTCCCACTACTCTTTGTC 3' and 5' TAAGCCTCAGGGTACTTATC 3'; GPX3, 5' GACCAGGTGGAGGCTTTG 3' and 5' GAGGTGGGAGGACAGGAG 3'; GAPDH, 5' AATCCCAT CACCATCTTC 3' and 5' AGGCTGTTGTCA TACTTC 3'. Each assay was repeated in triplicate.

### 2.8 Co-Immunoprecipitation (co-IP) and Western Blot Assays

The co-IP assay was used to examine the interaction between TRIM6 and GPX3. The experimental workflow was described in a previous report [21]. Cell lysates were incubated for 1 h at 4 °C with anti-TRIM6 (#Orb623826, Biorbyt, Cambridge, MA, USA), anti-GPX3 (Abcam, Ab275965, Cambridge, MA, USA), or control IgG (#sc-2748, Santa Cruz Biotech, Santa Cruz, CA, USA), followed by protein A/G-agarose for 3 h at 4 °C. Precipitates were washed three times in lysis buffer and then evaluated by Western blot analysis.

The procedure for Western blot analysis was described in our previous report [9]. After blocking with 5% skim milk, the membranes were incubated overnight at 4 °C with primary antibodies against active IL-1β (#63124, CST, Danvers, MA, USA), IL-1β (#16806-1-AP, Proteintech, Wuhan, China), Caspase-1 (#Ab179515, Abcam, Cambridge, MA, USA), cleaved Caspase-1 (#89332, CST), TRIM6 (#11953-1-AP, Proteintech), GPX3 (#Ab256470, Abcam), NLRP3 (#Ab263899, Abcam), GSDMD (#AF4013, Affinity Biosciences, Cincinnati, OH, USA), GSDMD-N (#Ab215203, Abcam), or GAPDH (#60004-1-Ig, Proteintech), follow by incubation with a secondary antibody (#A0208, #A0216, Beyotime, Shanghai, China). The enhanced chemiluminescence system (#WBULS0100, Millipore, Billerica, MA, USA) was used to reveal the band intensity, and Image J software for densitometry. The supernatants were used for detecting the active forms of IL-1β, and the lysates were used for detecting other protein levels. Each assay was repeated in triplicate.



**Fig. 1. Angiotensin II-induced ROS production is mediated by TRIM6.** Transfection of HK2 cells was performed with TRIM6-interfering lentivirus or with control virus for 24 h. The cells were then treated with 1  $\mu$ M Ang II for 24 h. (A) TRIM6 protein expression. (B) TRIM6 mRNA expression. (C,D) Flow cytometry detection of ROS. (E–G) Biochemical detection of MDA, GSH, and SOD. \*\*\* $p < 0.001$  vs. shNC; ^^ $p < 0.001$  vs. Ang II + shNC. NC, Normal Control; ROS, reactive oxygen species; TRIM6, Tripartite motif 6; HK2 cells, human kidney-2 cells; MDA, malondialdehyde; GSH, glutathione; SOD, Superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DCFH-DA, 2',7'-Dichlorodihydrofluorescein diacetate.

### 2.9 Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 7.0 (Graphpad, La Jolla, CA, USA). The normality of data was assessed and found to obey normal distribution characteristics. Data was presented as the mean  $\pm$  standard deviation (SD) from at least three independent experiments. Student's *t*-test was used for comparison of two groups, and analysis of variance (ANOVA) was used for comparison of more than two groups.  $p < 0.05$  was considered significant.

## 3. Results

### 3.1 Silencing of TRIM6 Inhibits Ang II-Induced ROS Generation

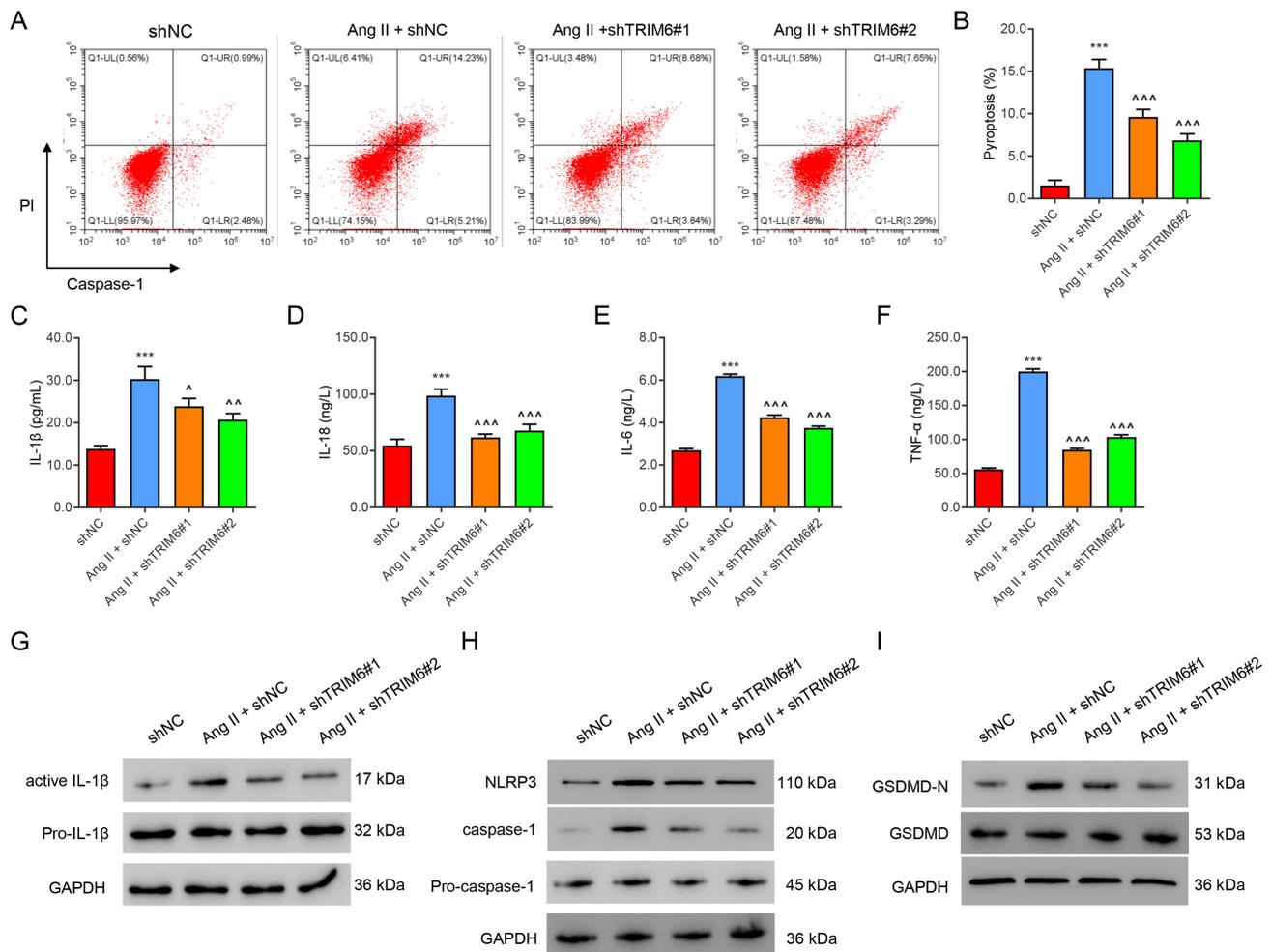
To study the role of TRIM6 in ROS production, TRIM6 expression in HK2 cells was silenced with lentivirus, or induced by Ang II (1  $\mu$ M) treatment for 24 hours (Fig. 1A,B). Ang II significantly increased the levels of ROS (Fig. 1C,D) and MDA (Fig. 1E), but decreased the levels of GSH (Fig. 1F) and SOD (Fig. 1G). All of these changes were ameliorated by the silencing of TRIM6 expression. These results indicate that Angiotensin II-induced ROS production was mediated by TRIM6.

### 3.2 Silencing of TRIM6 Inhibits Angiotensin II-induced Inflammation and Pyroptosis

To study the role of TRIM6 in pyroptosis, HK2 cells in which TRIM6 was silenced were challenged with Ang II (1  $\mu$ M) for 24 hours. Flow cytometry results showed that Ang II significantly promoted the pyroptosis of HK2 cells (Fig. 2A,B) and increased the levels of the inflammatory factors IL-1 $\beta$  (Fig. 2C), IL-18 (Fig. 2D), IL-6 (Fig. 2E), and TNF- $\alpha$  (Fig. 2F). Immunoblotting results revealed that Ang II markedly up-regulated the pyroptosis marker proteins NLRP3, GSDMD-N, and active Caspase-1 (Fig. 2G–I). In contrast, these Ang II-mediated effects were all significantly abolished by the silencing of TRIM6. This suggests that Angiotensin II-induced pyroptosis and inflammation are mediated by TRIM6.

### 3.3 NAC Suppresses TRIM6-Induced ROS Production and Pyroptosis

Next, TRIM6 was successfully over-expressed in HK2 cells, which were then treated with the ROS scavenger NAC (100  $\mu$ M) for 24 h. Overexpression of TRIM6 markedly up-regulated the levels of ROS (Fig. 3A,B) and MDA (Fig. 3C), but decreased the levels of GSH and SOD (Fig. 3D,E). Overexpression of TRIM6 also sharply increased the level of pyroptosis (Fig. 3F,G) and the lev-



**Fig. 2. Ang II-induced inflammation and pyroptosis are mediated by TRIM6.** HK2 cells were transfected with TRIM6-interfering lentivirus or control virus for 24 h, then treated with 1  $\mu$ M of Ang II for 24 h. (A,B) Flow cytometric assay of pyroptosis. (C–F) ELISA assay of IL-1 $\beta$ , IL-18, IL-6 and TNF- $\alpha$ . (G–I) Western blots of pyroptotic protein expression. \*\*\* $p$  < 0.001 vs. shNC;  $^{\wedge}$  $p$  < 0.05,  $^{\wedge\wedge}$  $p$  < 0.01,  $^{\wedge\wedge\wedge}$  $p$  < 0.001 vs. Ang II + shNC. ELISA, Enzyme-Linked Immunosorbent Assay; NLRP3, Nucleotide-binding domain leucine-rich repeat and pyrin domain-containing protein 3; GSDMD-N, Gasdermin-N Domain.

els of IL-1 $\beta$  (Fig. 3H) and IL-18 (Fig. 3I). Western blot analysis revealed that TRIM6 overexpression markedly up-regulated the pyroptosis-related markers NLRP3, active Caspase-1 (P10), and GSDMD-N (Fig. 3J,K). These effects of TRIM6 overexpression were all significantly abolished by NAC, indicating the importance of ROS in TRIM6-induced pyroptosis.

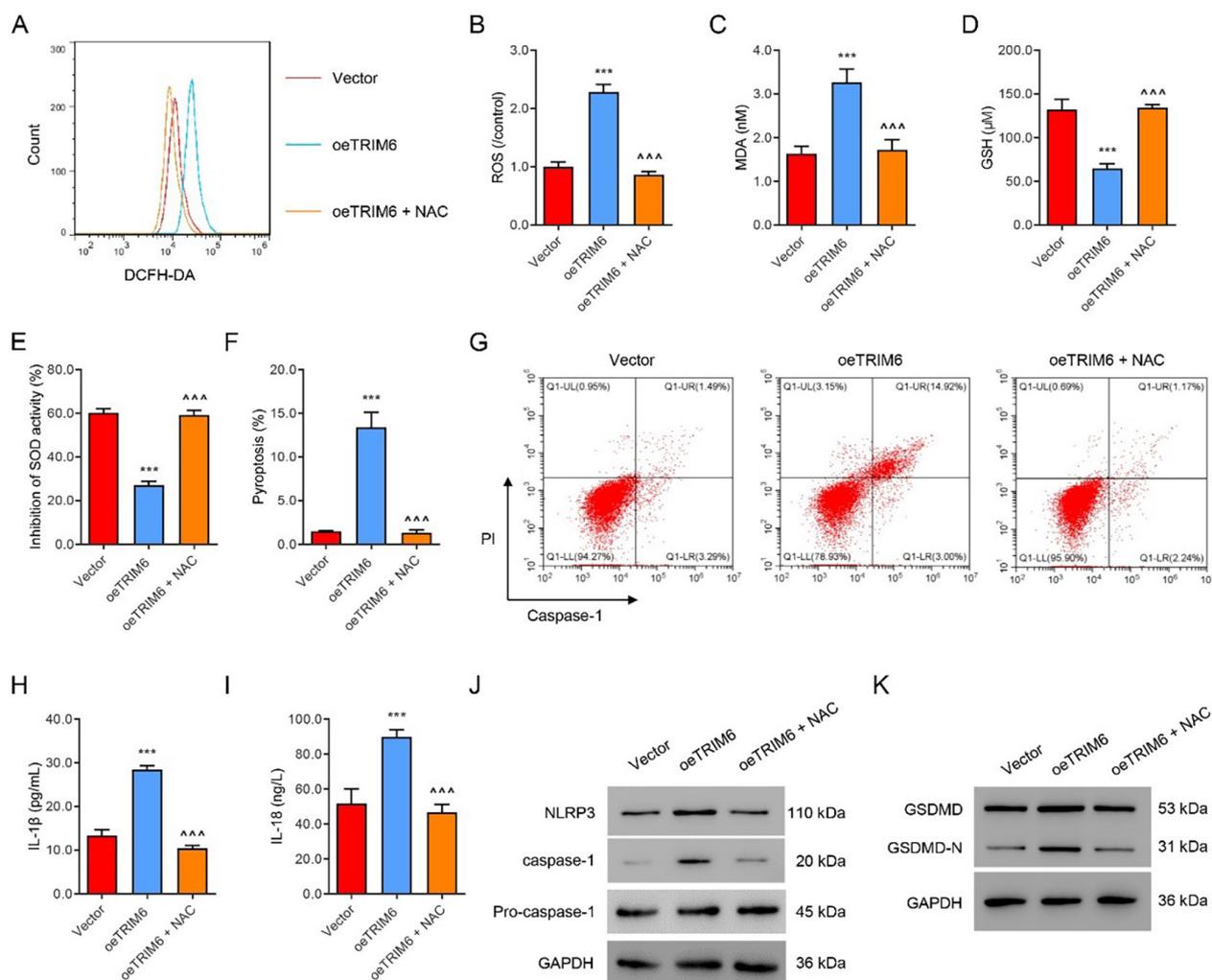
### 3.4 ROS Induces HK2 Cell Pyroptosis by Activating NLRP3

We further investigated the roles of Ang II-mediated ROS production and the NLRP3 signal in inflammasome activation and pyroptosis. The ROS scavenger NAC and the NLRP3 agonist BMS-986299 (1  $\mu$ M) were added to Ang II-treated HK2 cells. Ang II was found to induce ROS production, inflammation, and pyroptosis, which were all reduced by NAC treatment (Fig. 4A–D). However, co-treatment with BMS-986299 abolished the effects of NAC,

except for the ROS level. Immunoblotting results revealed that Ang II notably up-regulated expression of the pyroptosis marker proteins active Caspase-1, active IL-1, and GSDMD-N (Fig. 4E,F). These Ang II-mediated effects were all significantly reduced by NAC treatment, while co-treatment with BMS-986299 abolished the effects of NAC on protein expression (Fig. 4E,F).

### 3.5 TRIM6 Interacts with GPX3 and Promotes its Ubiquitination in HK2 Cells

The expression of GPX3 was evaluated to gain a better understanding of TRIM6 function in this model. TRIM6 overexpression was found to significantly decrease the level of GPX3 protein. Silencing of TRIM6 significantly increased the level of GPX3 protein, but neither TRIM6 overexpression or silencing affected GPX3 at the mRNA level (Fig. 5A). The decrease in GPX3 level with TRIM6 overexpression was suppressed by the proteasome inhibitor



**Fig. 3. NAC suppresses TRIM6-induced ROS production and pyroptosis.** TRIM6-overexpressing HK2 cells were treated with the ROS inhibitor NAC (100 μM) or control solvent for 24 h. (A,B) Flow cytometry evaluation of ROS. (C–E) Biochemical evaluation of MDA, GSH, and SOD. (F,G) Flow cytometry evaluation of pyroptosis. (H,I) ELISA evaluation of IL-1β and IL-18. (J,K) Western blot evaluation of pyroptosis marker protein expression. \*\*\* $p < 0.001$  vs. vector; ^^^ $p < 0.001$  vs. TRIM6 overexpression (oeTRIM6). NAC, N-acetyl-L-cysteine.

MG132 (Fig. 5B). Co-IP results showed that TRIM6 interacted with GPX3 (Fig. 5C). Importantly, the overexpression of TRIM6 in HK2 cells significantly promoted the ubiquitination of GPX3 (Fig. 5D). Together, these results demonstrate that TRIM6 interacts with GPX3 and promotes its ubiquitination.

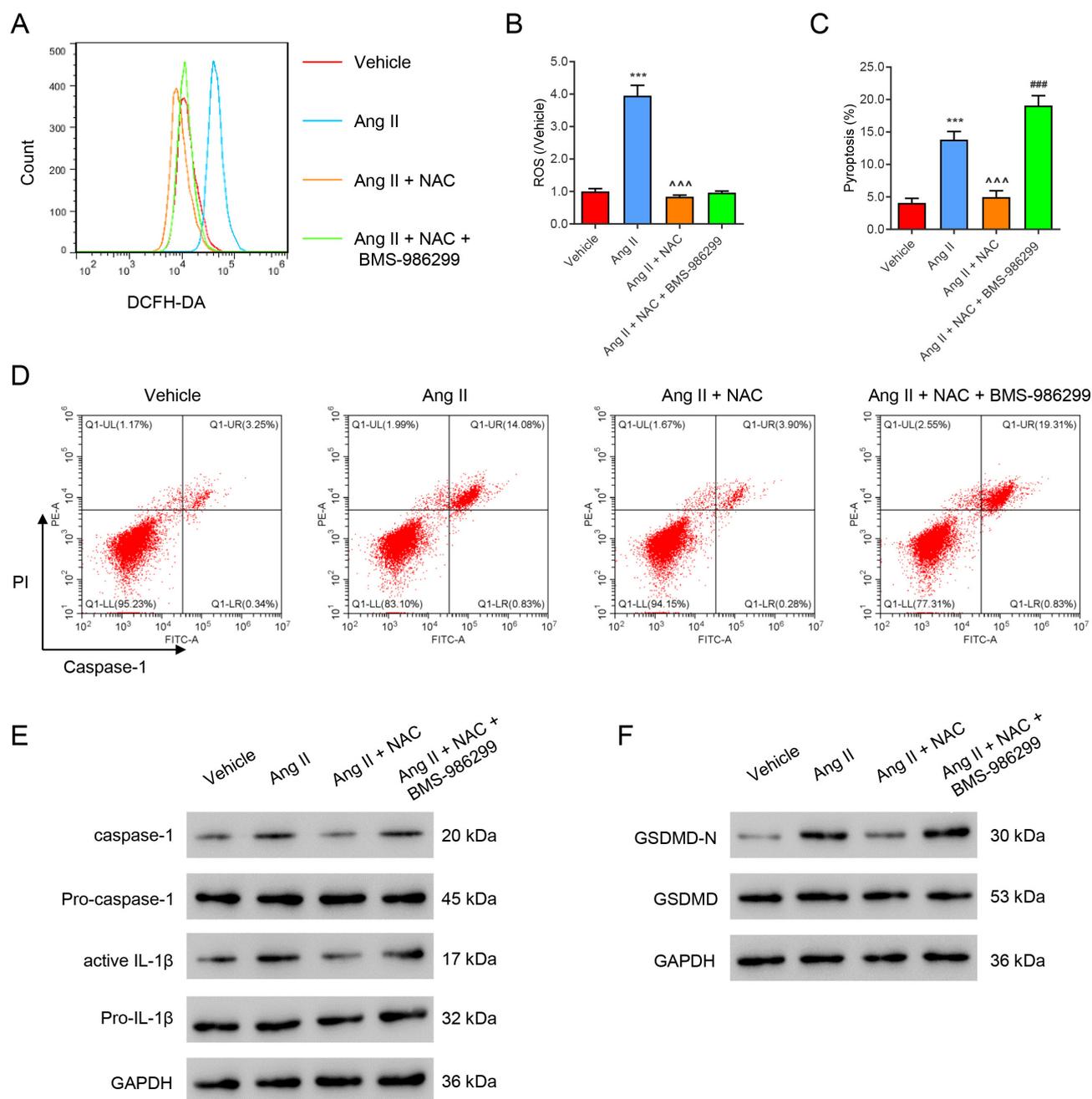
### 3.6 GPX3 Inhibits TRIM6-Induced ROS Production and Pyroptosis

GPX3 was successfully overexpressed in HK2 cells that overexpress TRIM6 (Fig. 6A). The overexpression of GPX3 significantly abolished TRIM6-induced ROS production (Fig. 6B,C) and MDA content (Fig. 6D). Overexpression of GPX3 also attenuated the decreased GSH level (Fig. 6E) and SOD level (Fig. 6F) associated with TRIM6

overexpression. Moreover, overexpression of GPX3 reversed the following changes induced by TRIM6 overexpression: increased pyroptosis (Fig. 6G,H), IL-1β (Fig. 6I), and IL-18 (Fig. 6J), and increases in the pyroptosis-related marker proteins NLRP3, GSDMD-N, and active Caspase-1 (Fig. 6K,I,L). Together, these results indicate that GPX3 inhibits TRIM6-induced ROS production and pyroptosis.

## 4. Discussion

The inappropriately activated renin-angiotensin system (RAS) plays a critical role in renal tissue injury and remodeling in CKD progression. Ang II mediates the majority of the classical biological functions of the RAS system and regulates renal cellular and physiological responses in the renal system [22]. Ang II is also related to renal in-

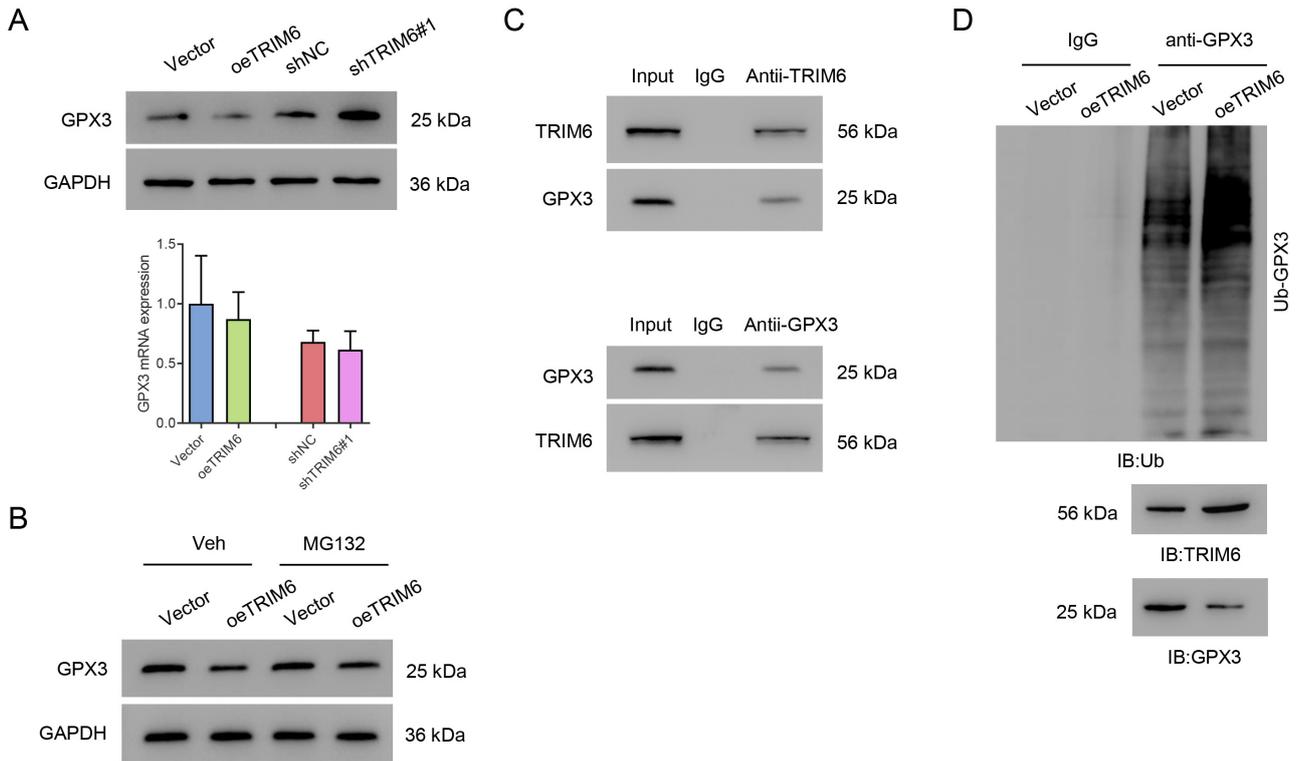


**Fig. 4. ROS induces HK2 cell pyroptosis by activating NLRP3.** Ang II-treated HK2 cells were incubated with the ROS inhibitor NAC (100  $\mu$ M) or/and BMS-986299 (1  $\mu$ M) for 24 h. (A,B) Flow cytometry evaluation of ROS. (C,D) Flow cytometry evaluation of pyroptosis. (E,F) Western blot evaluation of pyroptosis marker protein expression.  $***p < 0.001$  vs. vehicle;  $^^p < 0.001$  vs. Ang II treatment,  $###p < 0.001$  vs. Ang II + NAC treatment. FITC-A, Fluorescein isothiocyanate-A; BMS-986299 is NLRP3 agonist.

jury and fibrosis via activating renal tubular cells as well as local immune cells to increase inflammation release, such as IL-6, and TNF- $\alpha$ , and causes cell apoptosis [23,24]. Ang II is widely used to treatment renal cells *in vitro* to detected the pathobiology of CKD [25,26]. In the present study, we first revealed that Ang II induced the pyroptosis of HK2 cells by markedly increasing the ROS level via TRIM6. Administration of the ROS scavenger NAC significantly ameliorated Ang II-induced ROS and pyroptosis in HK2 cells.

Furthermore, the NLRP3 agonist BMS-986299 abolished the effects of NAC, but did not affect the ROS level. Mechanistic studies indicate that the functional role of TRIM6 in the Ang II-induced ROS changes and pyroptosis of HK2 cells is via the ubiquitination of GPX3.

ROS-mediated redox signaling plays a very important role in various biological processes. Redox balance is critical, as elevated ROS levels may damage cells [27]. The accumulation of ROS also has been shown to trigger an in-

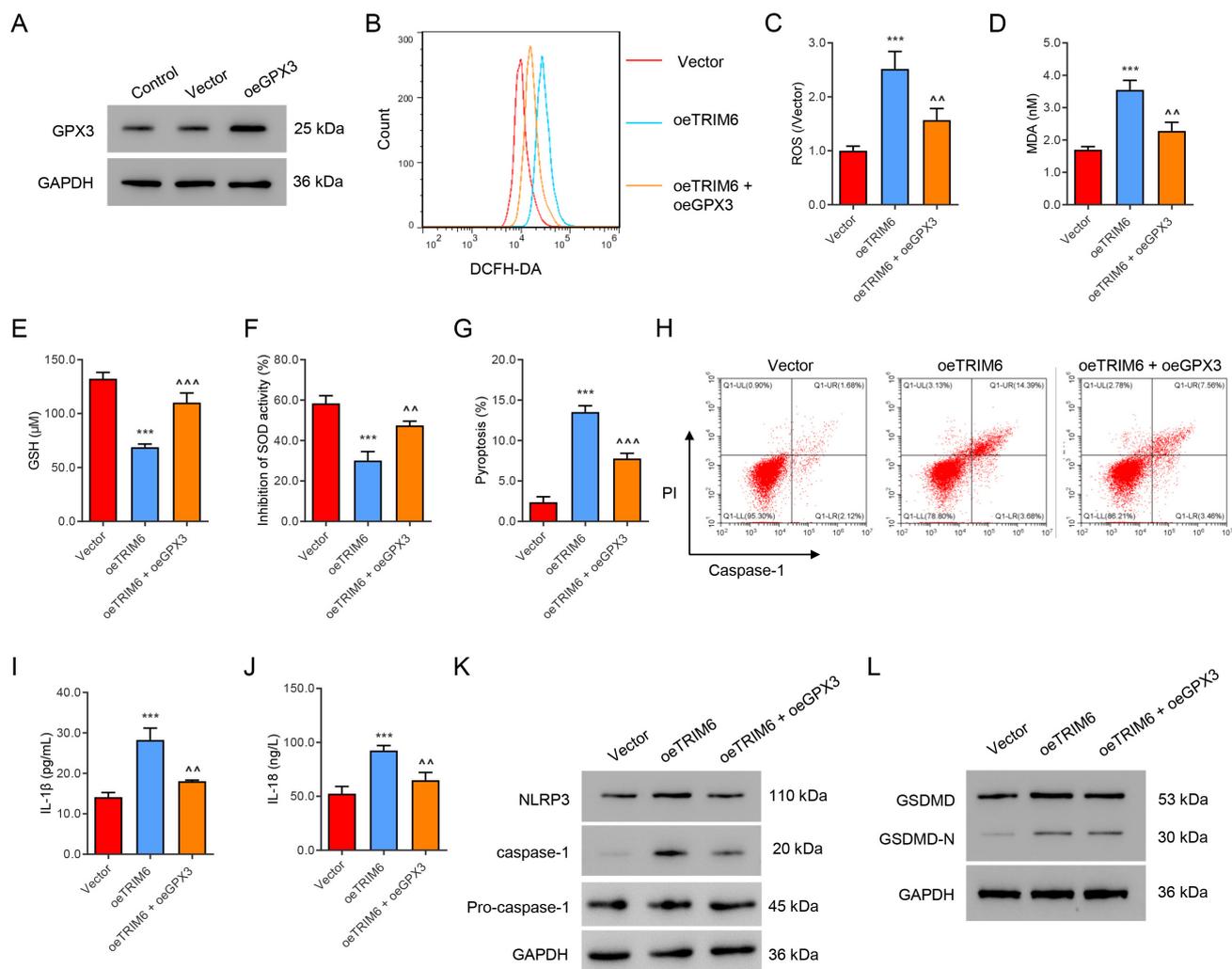


**Fig. 5. TRIM6 promotes GPX3 ubiquitination in HK2 cells.** (A,B) Western blot (upper panel) and qPCR (lower panel) were used to evaluate GPX3 expression after the silencing or overexpression of TRIM6 in HK2 cells. (C) TRIM6-overexpressing HK2 cells were treated with 10  $\mu$ M MG132 for 4 h and Western blot was then used to evaluate GPX3. (D) Co-IP assay detects the ubiquitination of GPX3. GPX3, Glutathione peroxidase 3; Co-IP, Co-Immunoprecipitation.

flammatory response [7]. Furthermore, ROS-induced activation of the NLRP3 inflammasome pathway has been reported in triple-negative breast cancer [28]. Activation of the NLRP3 inflammasome can regulate IL-1 $\beta$  and IL-18 levels via the NF- $\kappa$ B pathway [29], while increases in NLRP3 and caspase-1 expression favor the progression of CKD and RF [29]. Inflammasome activation requires two signals, so called priming and activation [30]. Priming is triggered by microbial molecules or endogenous cytokines and leads to the upregulation of NLRP3 and pro-IL-1 $\beta$  through the activation of transcriptional factor NF- $\kappa$ B. After receiving the priming signal, NLRP3 inflammasomes are activated by various factors such as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) [31]. Some natural products such as dihydroquercetin have shown efficacy at attenuating RF via the suppression of ROS and the NLRP3 inflammasome [32]. Our results indicate that overexpression of TRIM6 significantly increased ROS levels, decreased GSH and SOD, and promoted inflammasome activation in HK2 cells. These findings indicate that TRIM6 promotes ROS-dependent inflammasome activation and pyroptosis in HK2 cells.

Pyroptosis is one of the main forms of programmed cell death and serves as a defensive barrier against endogenous and exogenous pathogens [33]. As mentioned above,

ROS can activate the NLRP3 inflammasome, resulting in the production and release of pro-inflammatory cytokines. Activation of the NLRP3 inflammasome also leads to the cleavage of gasdermin D (GSDMD). This causes release of its N-terminus and ultimately the formation of pores on the cell membrane, resulting in cell rupture and the release of cytokines [29]. Pyroptosis has been demonstrated to regulate numerous biological activities. For instance, increased expression of pyroptosis-related proteins has been associated with a lower risk of breast cancer [34]. Inhibition of GSDMD-dependent pyroptosis reduces the progression of silica-induced pulmonary inflammation and fibrosis [35]. Pyroptosis was also found to be involved in renal interstitial fibrosis in the unilateral ureteral occlusion model [36]. Another study found that renal tubule injury induced by ureteral obstruction is highly susceptible to the initiation of fibrosis, with pyroptosis playing a major part in this process [37]. The present results showed that Angiotensin II significantly promoted pyroptosis, as shown by increased levels of IL-1 $\beta$  and IL-18, while also up-regulating the expression of pyroptosis marker proteins including NLRP3, GSDMD-N, and active Caspase-1 (P10). More importantly, the present findings suggest the promotion of pyroptosis was dependent on TRIM6. TRIM6 was found to perform an important regulatory function in pyroptosis-mediated HK2 cell injury.



**Fig. 6. GPX3 mediates ROS production and pyroptosis induced by TRIM6.** (A) Cells were infected with oeGPX3 virus or control vector, and GPX3 expression was evaluated by Western blot assay. (B,C) Flow cytometry evaluation of ROS. (D-F) Biochemical evaluation of MDA, GSH, and SOD. (G,H) Flow cytometry evaluation of pyroptosis. (I,J) ELISA evaluation of IL-1 $\beta$  and IL-18. (K,L) Western blot measurement of pyroptosis marker protein expression. \*\*\* $p < 0.001$  vs. vector; ^^ $p < 0.01$ , ^^ $p < 0.001$  vs. TRIM6 overexpression.

GPX3 has important antioxidant activity. It uses glutathione to catalyze the reduction of intracellular ROS, thereby protecting cells, DNA, and proteins against ROS damage [38]. Studies have shown that GPX3 overexpression exerts anti-oxidative stress protection against acute kidney injury, and improves the viability of tubular cells [39]. On the other hand, decreased GPX3 expression diminished its anti-oxidative stress effects and activated pro-tumorigenic redox signaling, thereby promoting tumor growth, proliferation, and metastasis [40]. GPX3 also participates in fibrosis, and oxidative stress is considered a regulator in the development of idiopathic pulmonary fibrosis, which can be attributed to the upregulation of GPX3 [41]. The current study found that overexpression of TRIM6 significantly decreased the level of GPX3 protein, whereas the silencing of TRIM6 expression significantly increased the level of GPX3 protein. Furthermore, our results suggest

that TRIM6 decreased the GPX3 protein level by promoting its ubiquitination. These results suggest a new role for GPX3 in RF and broaden our understanding of the pathogenesis of RF.

Protein ubiquitination is a post-translational modification that regulates all aspects of cell activity, including the cell cycle, proliferation, apoptosis and differentiation [42]. Recently, TRIM proteins have aroused the interest of researchers due to their E3 ubiquitin ligase activity. For example, TRIM23 has been shown to regulate adipocyte differentiation via the ubiquitination of PPAR $\gamma$  [43]. TRIM6 was also reported to activate mTORC1 by ubiquitinating TSC1-TSC2 to promote endoplasmic reticulum stress and RF [9]. Our study demonstrates that TRIM6 facilitates GPX3 ubiquitination. More importantly, we show that TRIM6-regulated GPX3 ubiquitination modulates RF by regulating ROS and pyroptosis. These findings demon-

strate the significance of the TRIM6/GPX3/pyroptosis axis in RF, and may help with new drug development for RF/CKD.

It is worth noting that further research on TRIM6/GPX3-mediated RF will require the development of an animal model. *In vitro* studies with other cell types in addition to the HK-2 cell line should also provide more evidence. Although further experiments are necessary, the current findings highlight the importance of TRIM6/GPX3 in RF.

The main limitation of this experiment is the lack of *in vivo* experiments. Besides, Ang II could trigger various signaling, the present revealed the role of TRIM6/GPX3 in CKD *in vitro*. The protective role of TRIM6 might also associate with other signaling pathways, and we will explore this in future experiments.

## 5. Conclusions

These research findings provide novel insights into the role of TRIM6/GPX3 signaling in RF. They suggest that Ang II-induced ROS levels and pyroptosis in HK2 cells are mediated by TRIM6 via the promotion of GPX3 ubiquitination. Our findings shed light on the molecular mechanism of TRIM6/GPX3 in relation to RF, and may lead to the development of novel therapeutic options for RF and CKD.

## Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

Conceptualization, JL, LL, PT and QX. Methodology, LL, PT, WC and JH. Software, WC, WWL and WRL. Validation, QX, JC and WWL. Formal analysis, LL, JL and JH. Investigation, LL, JH and QX. Resources, JL. Data curation, JL, JC, WWL and WRL. Writing—original draft preparation, JL, LL, PT and QX. Writing—review and editing, all authors. Visualization, LL, WRL and JH. Supervision, JL. Project administration, JL. Funding acquisition, JL. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors contributed to editorial changes in the manuscript.

## Ethics Approval and Consent to Participate

The cellular study was reviewed and approved by Ethics committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (2019-AR-005).

## Acknowledgment

Not applicable.

## Funding

This work was supported by the National Natural Science Foundation of China (NSFC-82074261).

## Conflict of Interest

The authors declare no conflict of interest.

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