Received: 4 April 2019 | Accepted: 30 September 2019

DOI: 10.1002/jcp.29356

Journal of Cellular Physiology WILEY

ORIGINAL RESEARCH ARTICLE

MKL1 mediates TGF-β-induced CTGF transcription to promote renal fibrosis

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: 81670618, 81970638

Abstract

Aberrant fibrogenesis impairs the architectural and functional homeostasis of the kidneys. It also predicts poor diagnosis in patients with end-stage renal disease (ESRD). Renal tubular epithelial cells (RTEC) can trans-differentiate into myofibroblasts to produce extracellular matrix proteins and contribute to renal fibrosis. Connective tissue growth factor (CTGF) is a cytokine upregulated in RTECs during renal fibrosis. In the present study, we investigated the regulation of CTGF transcription by megakaryocytic leukemia 1 (MKL1). Genetic deletion or pharmaceutical inhibition of MKL1 in mice mitigated renal fibrosis following the unilateral ureteral obstruction procedure. Notably, MKL1 deficiency in mice downregulated CTGF expression in the kidneys. Likewise, MKL1 knockdown or inhibition in RTEs blunted TGF-β induced CTGF expression. Further, it was discovered that MKL1 bound directly to the CTGF promoter by interacting with SMAD3 to activate CTGF transcription. In addition, MKL1 mediated the interplay between p300 and WDR5 to regulate CTGF transcription. CTGF knockdown dampened TGF-β induced pro-fibrogenic response in RTEs. MKL1 activity was reciprocally regulated by CTGF. In conclusion, we propose that targeting the MKL1–CTGF axis may generate novel therapeutic solutions against aberrant renal fibrogenesis.

KEYWORDS
epigenetics, renal fibrosis, transcription factor, transcriptional regulation

1 | INTRODUCTION

The kidneys are a major excretory and endocrine organ in humans. It plays versatile roles to maintain the homeostasis. Pathogens, toxic substances, and systemic/local hemodynamic disturbance interfere with the normal functions of the kidneys. When the kidneys are incapacitated in patients with chronic kidney disease devastating consequences ensue, which without effective intervention inevitably lead to renal failure (Schlaich et al., 2009). Renal fibrosis, taking place in both the glomerular and the interstitial regions, is considered one of the direst consequences in patients with end-stage renal disease (ESRD). Renal fibrosis disrupts the renal architecture and promotes the loss of renal functions (Eddy, 1996). It remains debatable where activated myofibroblast, the major cell type responsible for fibrogenesis, is originated in the fibrotic kidneys (Zeisberg & Neilson, 2010). Renal tubular epithelial cells can trans-differentiate into myofibroblast in part by undergoing a conserved process known as epithelial-to-mesenchymal transition, or EMT (Y. Liu, 2010).

Connective tissue growth factor (CTGF), also known as CCN2, is a matricellular protein that has been documented to regulate a wide range of physiological and pathological processes (Phanish, Winn, & Dockrell, 2010). CTGF expression levels are elevated in the kidneys in different mouse models of renal fibrosis and in human biopsy specimens from patients with chronic kidney diseases (Ito et al., 1998; Nguyen et al., 2008; Yokoi et al., 2002). More important, there

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appears to be a positive correlation between CTGF levels and the severity of renal fibrosis (Lipson, Wong, Teng, & Spong, 2012). CTGF expression is upregulated in renal tubular epithelial cells (RTECs) by transforming growth factor (TGF-β; Wang, Denichilo, Brubaker, & Hirschberg, 2001), glucose (X. Liu, Luo, Pan, Wu, & Chen, 2007), endothelin (Recchia et al., 2009), and angiotensin II (Ruperez et al., 2003). TGF-β activates CTGF expression by relaying the pro-fibrogenic signal to the SMAD family of transcription factors, which in turn bind to the conserved SMAD binding element (SBE) located on the proximal CTGF promoter and activate CTGF transcription (Holmes et al., 2001). Accordingly, mice with a deficiency in SMAD3 are partially resistant to renal fibrosis accompanying downregulation of CTGF in the kidneys (Inazaki et al., 2004). Conversely, genetic deletion of SMAD7, the inhibitory SMAD, exacerbates renal fibrosis in mice with a concomitant increase in CTGF expression (Chung et al., 2009).

Megakaryocytic leukemia 1 (MKL1) is a transcriptional modulator known to bridge cytoskeletal reshuffling to stress response. MKL1 orchestrates a myriad of cellular responses to environmental and intrinsic stimuli including inflammation (Yu et al., 2014; Yu, Fang et al., 2017), oxidative stress (Yu et al., 2018), aging (Petrini et al., 2017), hypoxia (D. Chen et al., 2015; Yang et al., 2013), and mechanoresponse (Kuwahara et al., 2010). Mounting evidence portrays MKL1 as a key regulator of myofibroblast maturation and fibrogenesis (Small, 2012). MKL1 loss-of-function in mice attenuates myocardial infarction induced cardiac fibrosis (Small et al., 2010), bleomycin induced pulmonary fibrosis (Sisson et al., 2015), and carbon tetrachloride induced liver fibrosis (Tian et al., 2015). Morita, Mayanagi, and Sobue (2007) have previously demonstrated that MKL1 programs EMT in cultured RTECs in vitro. Elberg et al. (2008) have shown that activation of MKL1 by TGF-β in human RTECs induces the transcription of α-SMA, a marker of activated myofibroblast. More recently, Muehlich, Rehm, Ebenau, and Goppelt-Struebe (2017) have reported that dynamic interplay between MKL1, YAP/TAZ, and SMADs in human RTECs mediates synergistic induction of CTGF transcription by TGF-β and cytochalasin D. In the present study we investigated the role of MKL1 in CTGF transcription and renal fibrosis. We report that MKL1 deletion or inhibition blocks unilateral ureteral tract obstruction (UUO)-induced renal fibrosis in mice by contributing to epigenetic activation of CTGF transcription. CTGF, in turn, regulates TGF-β induced fibrogenic response in RTECs by promoting nuclear translocation of MKL1.

2 | MATERIALS AND METHODS

2.1 | Cell culture, plasmids, transient transfection, and reporter assay

MKL1 expression constructs (Z. Li, B. Chen, W. Dong et al., 2018), CTGF promoter constructs (Li et al., 2019), and ACTA2 promoter construct (Fan et al., 2015) have been described previously. Primary renal tubular epithelial cells were isolated and maintained in William's E media supplemented with 10% fetal bovine serum as previously described (Xu et al., 2015). Immortalized rat renal tubular epithelial cells (NRK-52E) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Small interfering RNAs were purchased from GenePharma Biotech. Transient transfections were performed with Lipofectamine 3000 or RNAiMax (Thermo Fisher Scientific). Cells were seeded 16–24 hr before transfection. On the day of transfection, plasmid DNA and Lipofectamine reagent were diluted in serum-free DMEM. After DNA–Lipofectamine complex had formed, it was added to each well dropwise. Luciferase activities were assayed 24–48 hr after transfection using a luciferase reporter assay system (Promega). Data were normalized by both protein concentration and GFP fluorescence and were expressed as relative luciferase unit (RLU) compared with the control group arbitrarily set as 1 (Fan et al., 2017; Yu, Li, Fang, & Xu, 2017).

2.2 | Protein extraction and western blot

Before harvesting, cells were washed two times with ice-cold phosphate-buffered saline (PBS) buffer. Cell pellet was obtained by spinning in a refrigerated centrifuge at 2,500 rpm for 10 min. Supernatant was discarded and cells was lyzed in ice-cold radio-immunoprecipitation assay (RIPA) buffer (1×PBS, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, and 0.5% sodium deoxycholate) supplemented with 100 μg/ml PMSF plus one protease inhibitor tablet (Roche, Mannheim, Germany) per 10 ml RIPA buffer as previously described (Z. Li, B. Chen, X. Weng et al., 2018). Typically, 50–100 μg of proteins were loaded and separated by 8% SDS-PAGE gel with all-blue protein markers (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 5% fat-free milk powder in Tris-buffered saline at room temperature for half an hour and then incubated with the following primary antibodies at 4°C overnight: anti-CTGF (Proteintech, 23936-1), anti-MKL1 (Santa Cruz, sc-32909), anti-α-SMA (Sigma, A5228), anti-α-tubulin (Proteintech, 11224-1), anti-Lamin B (Santa Cruz, sc-6216), and anti-β-actin (Sigma, A2228) antibodies.

2.3 | RNA isolation and Real-time PCR

RNA was extracted with a commercial RNAprep purification kit (Tiangen) as previously described (Z. Li, B. Chen, X. Weng et al., 2018). First-strand synthesis was carried out using a HiScript III RT SuperMix (Vazyme). Real-time PCR reactions were performed on an ABI Prism StepOne Plus system with a commercial Sybrgreen kit (Vazyme). Primers and Taqman probes used for real-time reactions were purchased from Shanghai Sangon Biotech.

2.4 | Mice

The animal experiments were conducted at Nanjing Medical University and the protocols were reviewed and approved by the Committee on Humane Treatment of Experimental Animals and Ethic

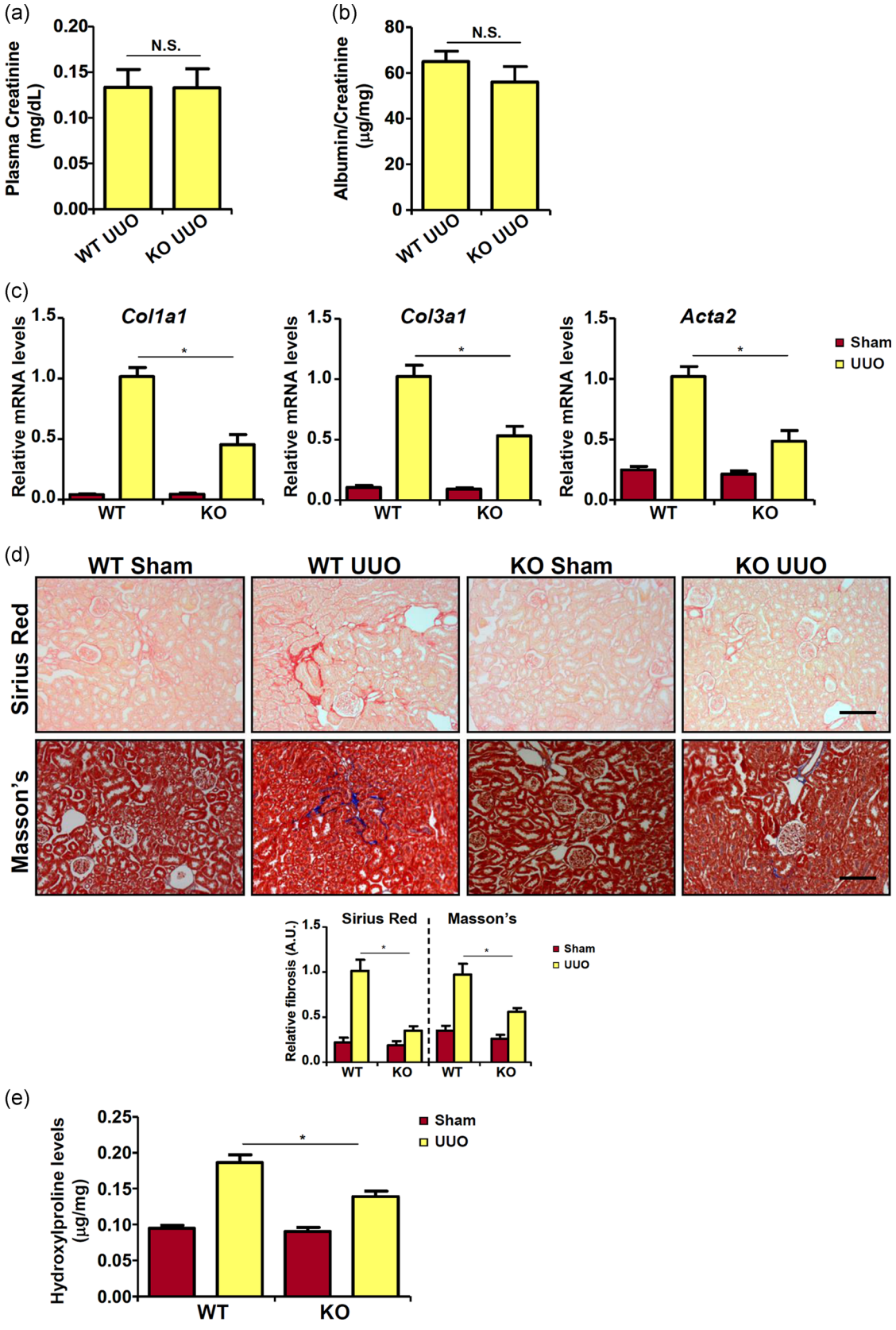

FIGURE 1 Continued.

Conduct of Animal Studies of Nanjing Medical University. Eight-week-old male MKL1 knockout mice (L. Liu et al., 2018) and wild type littermates were subjected to UUO or the sham surgery as previously described (Vielhauer et al., 2001). The mice were killed 2 weeks after the procedure. Plasma creatinine levels (Bioassays Systems) and proteinuria levels (Roche) were measured using commercially available kits per vendors' protocols with minor modifications. In certain experiments, CCG-203971 (Selleck) was injected peritoneally at a dose of 1 mg/kg every other day for 2 weeks following UUO.

2.5 | Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described before (Fan et al., 2017; N. Li, M. Kong et al., 2018; N. Li, M. Li et al., 2018; Shao et al., 2019; Weng et al., 2019; Yu, Li et al., 2017; Zeng et al., 2018; Zhang et al., 2018). In brief, chromatins were cross-linked with 1% formaldehyde for 15 min at room temperatures. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) supplemented with protease inhibitor tablet and PMSF. DNA was fragmented into ~500 bp pieces using a Diagenode Bioruptor sonicator. For each ChIP reaction, 100 μg of protein was incubated at 4°C overnight with 2 μg of the following antibodies: anti-MKL1 (Santa Cruz, sc-32909), anti-acetyl H3 (Millipore, 06–599), anti-trimethyl H3K4 (Millipore, 07–449), anti-p300 (Santa Cruz, sc-585), anti-WDR5 (Bethyl Laboratories, A302–429A), or anti-SMAD3 (Abcam, ab28379). For Re-ChIP, immune complexes were eluted with the elution buffer (1% SDS and 100 mM $NaCO_3$), diluted with the Re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris, pH 8.1), and incubated with a second antibody of interest. Released genomic DNA was phenol–chloroform extracted and precipitated by 100% ethanol followed by washing with 70% ethanol and allowed to air-dry for up to an hour. Dried DNA was dissolved in 50 μl deionized distilled water and 2–3 μl was used for each real-time PCR reaction. Serial dilutions of genomic DNA extracted from the same type of cells were included with ChIP samples as standards to calculate the amount of genomic DNA being precipitated by a particular antibody.

2.6 | Statistical analysis

One-way analysis of variance (ANOVA) with post hoc Scheffe's analyses were performed by SPSS software (IBM SPSS v18.0, Chicago, IL). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | MKL1 deficiency alleviates UUO-induced renal fibrosis in mice

First, we examined the effect of MKL1 deficiency on renal fibrosis in vivo in a mouse model of unilateral ureteral tract obstruction (Vielhauer et al., 2001); the mice were killed 2 weeks after the surgery for further examination. After the UUO procedure, both the MKL1 knockout (KO) mice and the wild type (WT) littermates displayed similar levels of renal injury as assessed by plasma creatinine levels (Figure 1a, WT: 0.136 ± 0.021 mg/dL; KO: 0.135 ± 0.021 mg/dL) and severity of proteinuria (Figure 1b, WT: 64.2 ± 4.5 μg/mg; KO: 58.5 ± 7.1 μg/mg), suggesting that MKL1 might play a nonessential role in UUO-induced disturbances of glomerular filtration function. In contrast, renal fibrosis was significantly attenuated in KO mice compared with WT mice because PCR quantification (Figure 1c) showed a downregulation collagen type I (*Col1a1* downregulated by 52%), collagen type III (*Col3a1* downregulated by 47%), and smooth muscle cell actin/α-SMA (*Acta2* downregulated by 56%) in the kidneys. Moreover, picrosirius red staining (KO vs. WT, downregulated by 61%) and Masson's trichrome staining (KO vs. WT, downregulated by 42%) both indicated that renal fibrosis was less extensive in KO mice than in WT mice (Figure 1d). Finally, measurements of renal hydroxylproline levels confirmed that MKL1 deletion resulted in a reduction of renal fibrosis in mice (Figure 1e, WT: 0.184 ± 0.014 μg/mg; KO: 0.147 ± 0.011 μg/mg).

Next, a well-documented compound (CCG-203971) was injected peritoneally in mice to inhibit MKL1 activity (Johnson et al., 2014; Sisson et al., 2015). CCG administration did not significantly alter glomerular infiltration as indicated by plasma creatinine levels (Figure 2a, vehicle: 0.158 ± 0.022 mg/dL; CCG-203971: 0.148 ± 0.031 mg/dL) and urine albumin levels (Figure 2b, vehicle: 73.6 ± 12.9 μg/mg; CCG-203971: 72.9 ± 11.6 μg/mg). Instead, CCG injection downregulated *Col1a1* by 62%, downregulated *Col3a1* by 74%, and downregulated *Acta2* by 31% in the kidneys (Figure 2c). CCG injection also decreased levels of collagenous tissues as assessed by picrosirius red (KO vs. WT, downregulated by 51%) and Masson's trichrome (KO vs. WT, downregulated by 34%) staining (Figure 2d) as well as renal hydroxylproline levels (Figure 2e, WT: 0.237 ± 0.021 μg/mg; KO: 0.175 ± 0.018 μg/mg). Together, these data demonstrate that either genetic deletion or pharmaceutical inhibition of MKL1 may mitigate UUO-induced renal fibrosis without altering glomerular filtration function in mice.

FIGURE 1 MKL1 deficiency alleviates UUO-induced renal fibrosis in mice. MKL1 knockout (KO) mice and wild type littermates were subjected to UUO to induce renal fibrosis. The mice were killed two weeks after the procedure. (a) Plasma creatinine levels were determined by chemiluminescence assay. (b) Urine albumin levels were determined by chemiluminescence assay. (c) Quantitative PCR was performed to determine the expression levels of *Col1a1*, *Col3a1*, and *Acta2*. (d) Paraffin sections were stained with picrosirius red and Masson's trichrome. Scale bar, 50 μm. (e) Hydroxylproline levels were determined by colorimetry. $N = 6$ mice for each group. PCR, polymerase chain reaction; UUO, unilateral ureteral tract obstruction

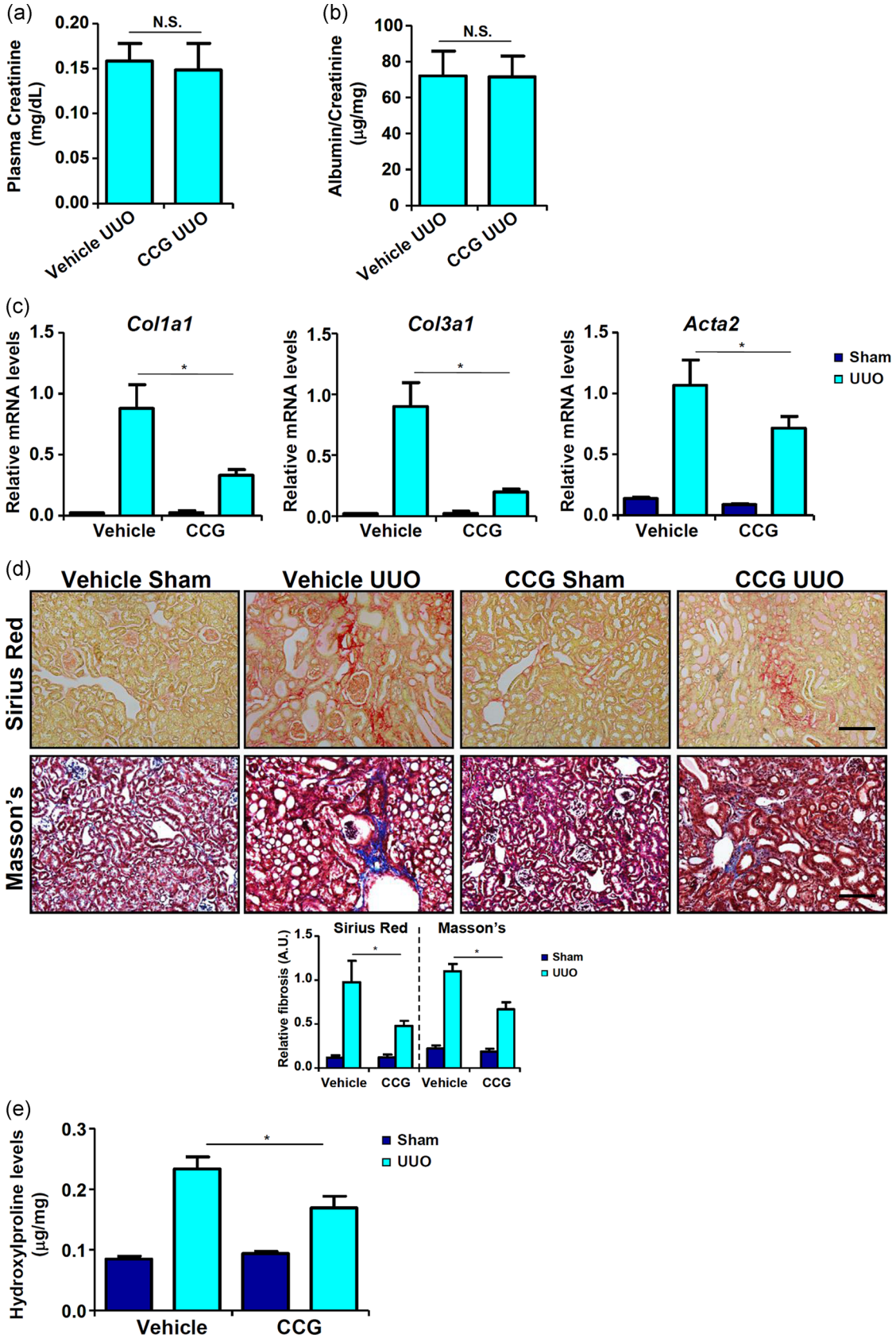

FIGURE 2 Continued.

3.2 | MKL1 is essential for CTGF induction in vitro and in vivo

CTGF is a pleiotropic growth factor with a well-documented role in renal fibrosis. As expected, CTGF levels were elevated in the kidneys in mice subjected to the UUO procedure (Figure 3a,b). The elevation of renal CTGF expression was not as high in MKL1 KO mice (KO UUO vs. KO sham, 2.3× upregulation) compared with the WT mice (WT UUO vs. WT sham, 4.5× upregulation), which indicated that MKL1 might be essential for CTGF induction. Similar experiments confirmed that UUO procedure increased renal CTGF messenger RNA (mRNA) expression by 9.4× in the vehicle-treated mice and by 6.9× in the CCG-treated mice (Figure 3c). Western blot analysis showed that CCG administration in mice reduced CTGF protein levels by 31% following UUO (Figure 3d). Next, we isolated primary renal tubular epithelial cells (RTECs) from MKL1 KO and WT mice. TGF-β treatment increased CTGF mRNA expression by 3.1× in WT RTECs but only by 1.8× in KO RTECs (Figure 3e). Induction of CTGF protein expression by TGF was also more modest in KO cells than in WT cells (Figure 3f, KO vs. WO, downregulated by 40%). Likewise, TGF-β-stimulated CTGF expression in primary RTECs (Figure 3g,h) was dose-dependently suppressed by CCG treatment: CCG at 5 μM downregulated CTGF induction by 27% whereas CCG at 10 μM downregulated CTGF induction by 49%. Together, these data suggest that MKL1 might contribute to TGF-β induced CTGF expression in vivo and in vitro.

3.3 | MKL1 interacts with SMAD3 to activate CTGF transcription

It was next determined whether MKL1 could directly activate CTGF transcription. We transfected into NRK-52E cells a CTGF promoter-luciferase construct with or without MKL1. Overexpression of MKL1 increased the CTGF promoter activity by up to 5.5× at the highest dose tested, which suggested that MKL1 might directly activate CTGF transcription (Figure 4a). It was also observed that MKL1 overexpression activated the CTGF promoter by 3.2× and TGF-β treatment activated the CTGF promoter by 1.7×; combined, MKL1 overexpression and TGF treatment synergistically activated the CTGF promoter by 4.8× (Figure 4b). On the contrary, a dominant negative (DN) form of MKL1, which lacks the trans-activation domain (Yu et al., 2018), diminished the activation of the CTGF promoter by TGF-β treatment by 37% (Figure 4c). It was noteworthy that when a conserved SMAD3 binding site within the CTGF promoter was mutated, MKL1 was unable to activate the CTGF promoter (Figure 4d), which indicated that MKL1 binding to the CTGF promoter might depend on SMAD3. Indeed, TGF-β treatment promoted the association of MKL1 with the region of the CTGF promoter containing the SMAD binding site (SBE) by 3.6× but not the distal CTGF promoter without this site (Figure 4f). Depletion of SMAD3 by siRNA (Figure 4e) significantly disrupted the association of MKL1 with the proximal CTGF promoter region by 54%. Re-ChIP suggested that TGF-β treatment also promoted the MKL1-SMAD3 interaction by 5.8× on the proximal CTGF promoter region (Figure 4g). Taken together, we conclude that MKL1 might be recruited by SMAD3 to activate CTGF transcription.

3.4 | MKL1 promotes epigenetic activation of CTGF transcription

Next, the epigenetic mechanism by which MKL1 regulates CTGF transcription was examined. When primary RTECs were treated by TGF-β, it was discovered that the CTGF proximal promoter was abounded with active histone markers, such as acetylated H3 (AcH3, Figure 5a, TGF treatment vs. no treatment, upregulated by 3.4×) and trimethylated H3K4 (H3K4Me3, Figure 5c, TGF treatment vs. no treatment, upregulated by 4.6×). Congruently, p300 (Figure 5b, TGF treatment vs. no treatment, upregulated by 2.9×), the acetyltransferase that catalyzes H3 acetylation, and WDR5 (Figure 5d, TGF treatment vs. no treatment, upregulated by 3.2×), a regulatory subunit of the COMPASS H3K4 trimethyltransferase complex, were detected on the CTGF proximal promoter after TGF-β was added to the cells. MKL1 deficiency, however, weakened the enrichment of AcH3 by 49% and the enrichment of H3K4Me3 by 50% probably through impairing the binding of p300 and WDR5 because p300 and WDR5 occupancies were downregulated by 40% and 45%, respectively, in the absence of MKL1. In addition, Re-ChIP assays provided evidence that TGF-β treatment augmented the interaction between p300 and WDR5 by 4.2× on the proximal CTGF promoter and MKL1 loss-of-function crippled this interaction by 53% (Figure 5e). It was similarly found that inhibition of MKL1 by CCG treatment diminished the accumulation of AcH3 (Figure 5f), H3K4Me3 (Figure 5h) by 51% and 60%, respectively. CCG treatment also impaired the recruitment of p300 (Figure 5g), WDR5 (Figure 5i) by 45% and 51%, respectively. Finally, CCG treatment disturbed the interaction between p300 and WDR5 surrounding the CTGF proximal promoter by 42% (Figure 5j). Altogether, these data suggest that MKL1 may contribute to CTGF transcription by coordinating the interplay between epigenetic factors on the CTGF promoter.

FIGURE 2 MKL1 inhibition attenuates UUO-induced renal fibrosis in mice. C57/BL6 mice were subjected to UUO to induce renal fibrosis. CCG injection (1 mg/kg) was administered every other day for 2 weeks. The mice were killed 2 weeks after the procedure. (a) Plasma creatinine levels were determined by chemiluminescence assay. (b) Urine albumin levels were determined by chemiluminescence assay. (c) Quantitative PCR was performed to determine the expression levels of *Col1a1*, *Col3a1*, and *Acta2*. (d) Paraffin sections were stained with picrosirius red and Masson's trichrome. Scale bar, 50 μm. (e) Hydroxylproline levels were determined by colorimetry. $N = 6$ mice for each group. PCR, polymerase chain reaction; UUO, unilateral ureteral tract obstruction

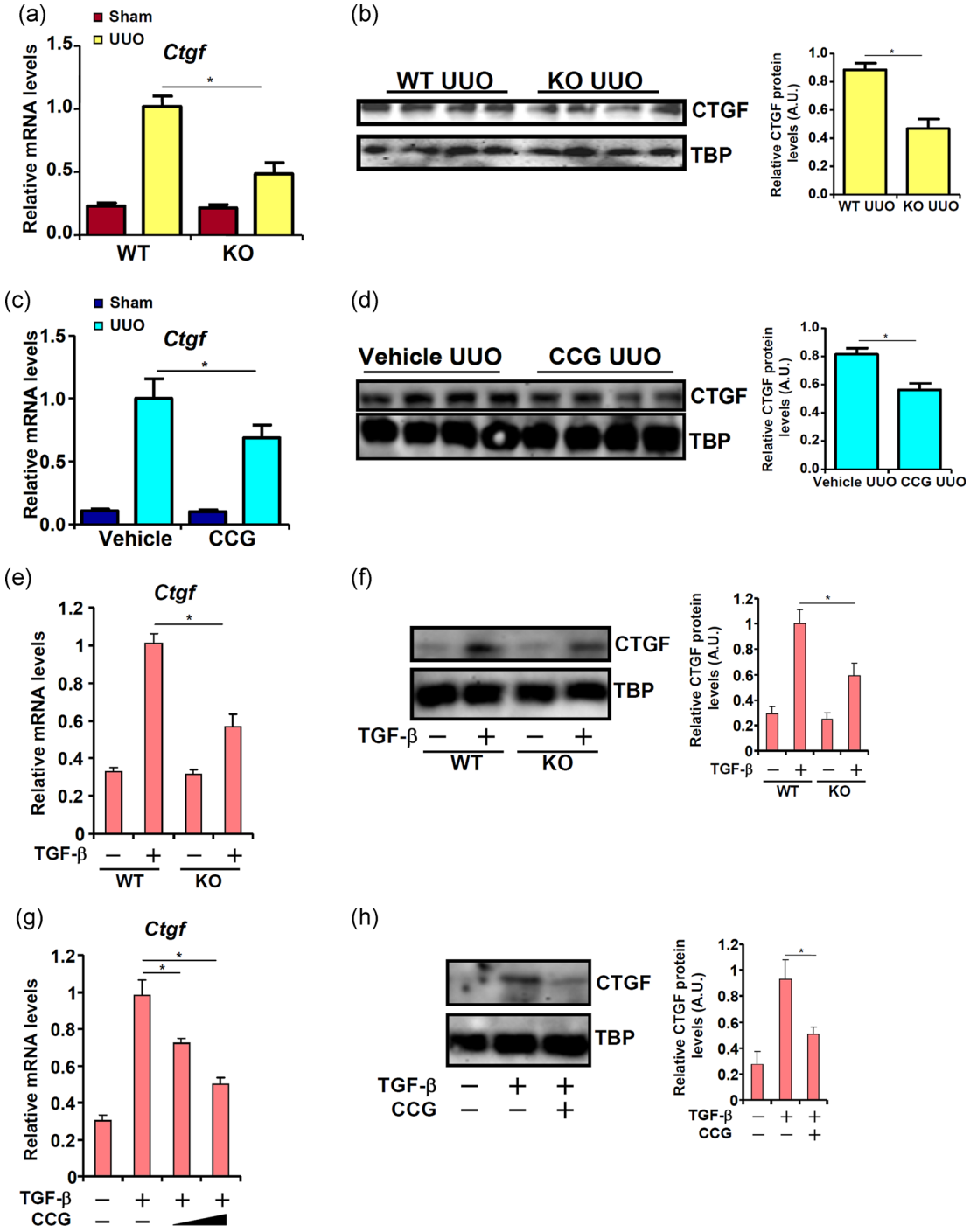

FIGURE 3 MKL1 is essential for CTGF induction in vitro and in vivo. (a and b) MKL1 knockout (KO) mice and wild type littermates were subjected to UUO to induce renal fibrosis. The mice were killed 2 weeks after the procedure. Quantitative PCR and western blot analysis were performed to determine CTGF expression. (c and d) C57/BL6 mice were subjected to UUO to induce renal fibrosis. CCG injection was administered every other day. The mice were killed 2 weeks after the procedure. Quantitative PCR and western blot analysis were performed to determine CTGF expression. (e and f) Renal tubular epithelial cells isolated from WT and KO mice were treated with TGF-β for 48 hr. Quantitative PCR and western blot analysis were performed to determine CTGF expression. (g and h) Primary renal tubular epithelial cells were isolated from WT mice and treated with TGF-β in the presence or absence of CCG (5 μM and 10 μM) for 48 hr. Quantitative PCR and western blot analysis were performed to determine CTGF expression. PCR, polymerase chain reaction; TGF-β, tumor necrosis factor-β; UUO, unilateral ureteral tract obstruction

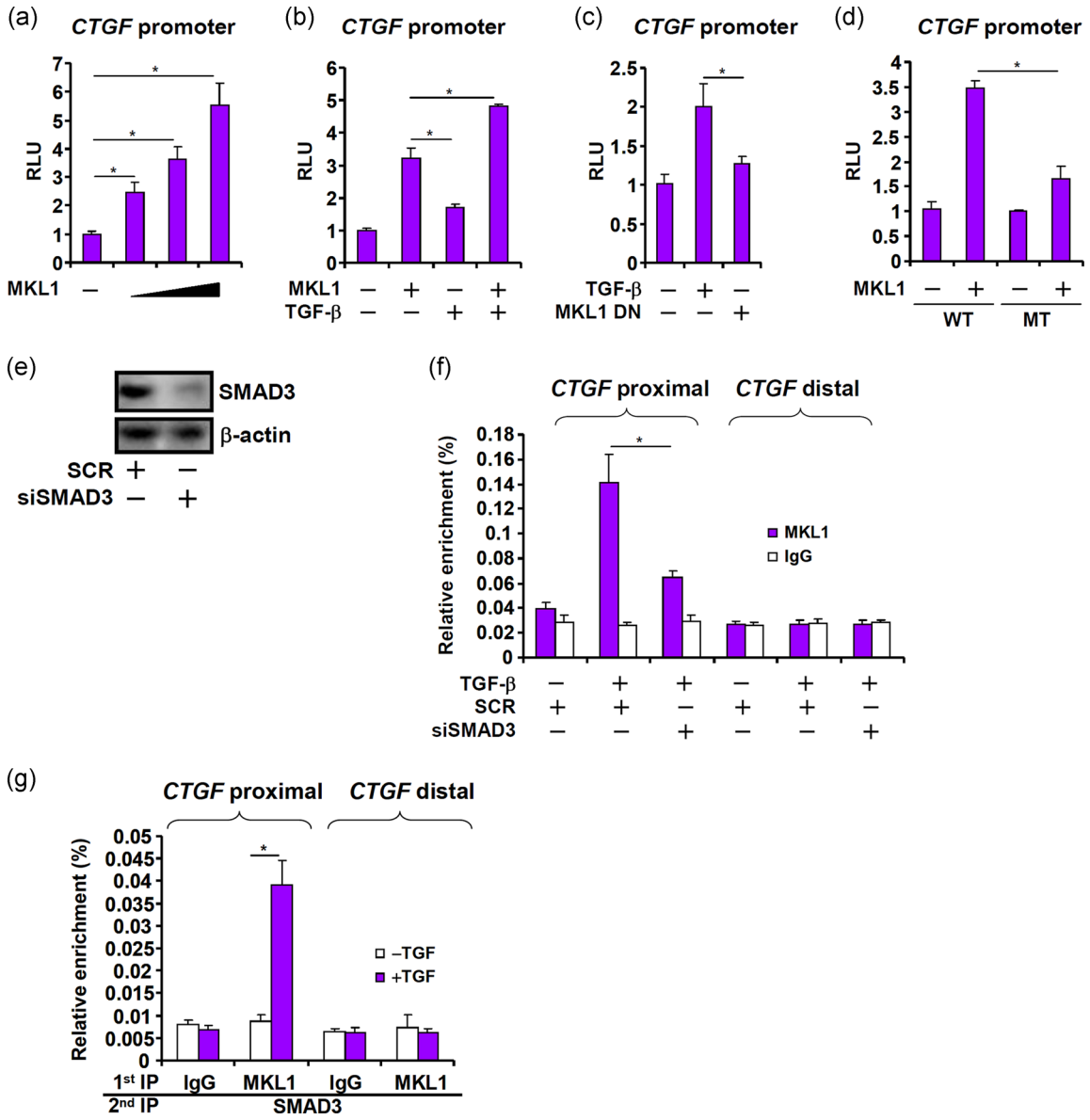

FIGURE 4 MKL1 interacts with SMAD3 to activate CTGF transcription. (a) A CTGF promoter-luciferase construct was transfected into NRK-52E cells with increasing doses of MKL1. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (b) A CTGF promoter-luciferase construct was transfected into NRK-52E cells with or without MKL1 followed by treatment with TGF-β. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (c) A CTGF promoter-luciferase construct was transfected into NRK-52E cells with or without dominant negative (DN) MKL1 followed by treatment with TGF-β. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (d) Wild type or SMAD mutant CTGF promoter-luciferase construct was transfected into NRK-52E cells with or without MKL1. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (e and f) Primary renal tubular epithelial cells were isolated from WT mice and transfected with small interfering RNA targeting SMAD3 or scrambled siRNA (SCR) followed by treatment with TGF-β. Knockdown efficiency was verified by western blot analysis. ChIP assay was conducted with anti-MKL1 or IgG. (g) Primary renal tubular epithelial cells were treated with TGF-β for 48 hr. Re-ChIP assay was conducted with indicated antibodies. ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; TGF-β, tumor necrosis factor-β

3.5 | CTGF is essential for TGF-β induced pro-fibrogenic transcription

Next, the issue whether CTGF could regulate TGF-β induced pro-fibrogenic transcription in RTECs was addressed. Knockdown of CTGF attenuated TGF-β induced mRNA levels of α-SMA by 55% and protein levels of α-SMA by 58% in NRK-52E cells (Figure 6a,b). CTGF knockdown also suppressed TGF-β induced trans-activation of the Acta2 promoter by 43% (Figure 6c). It was intriguing that CTGF silencing markedly impaired the binding of MKL1 to the Acta2 promoter region by 49% (Figure 6d). Because MKL1 can shuttle between the cytoplasm and the nucleus (Olson & Nordheim, 2010),

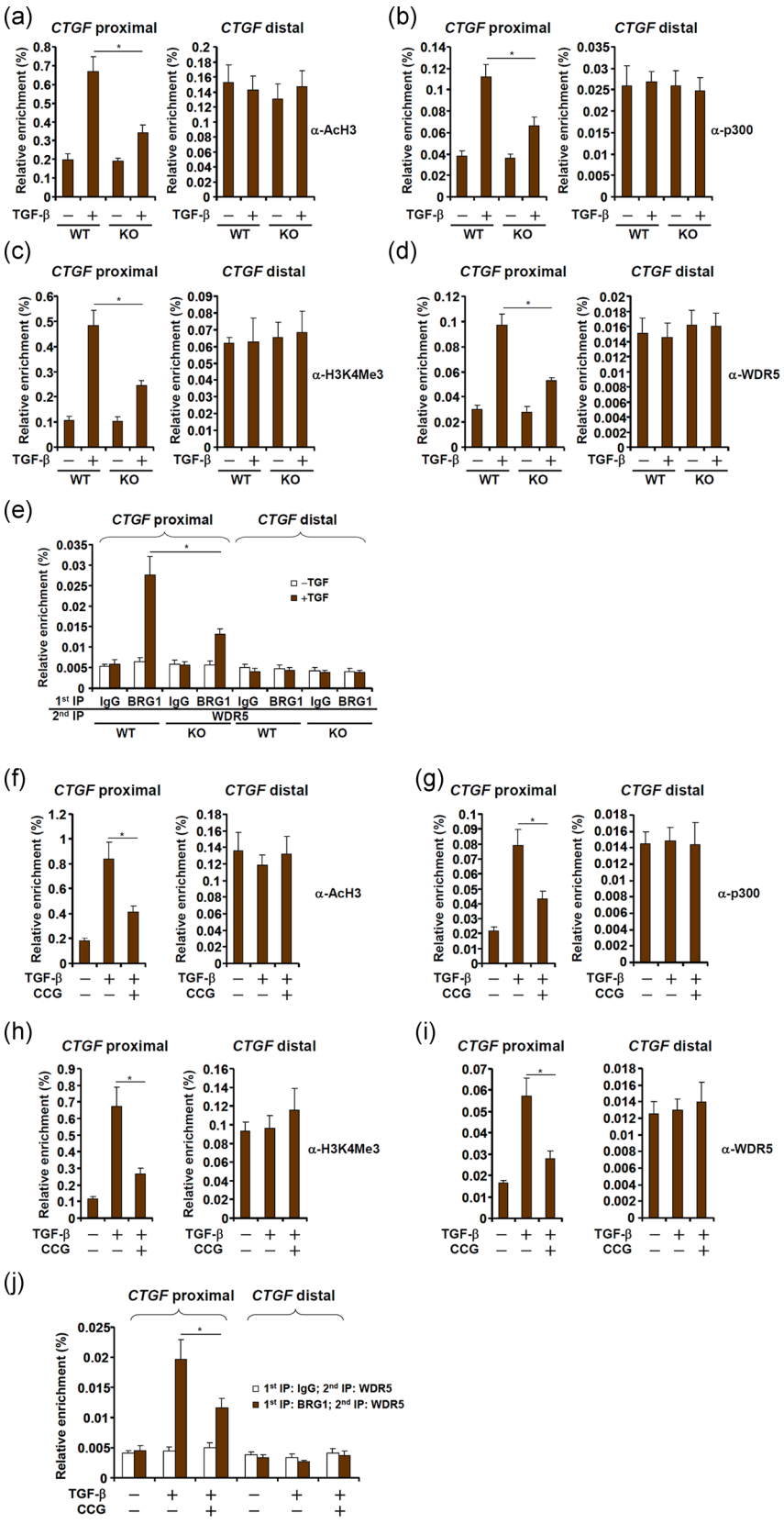

FIGURE 5 MKL1 promotes epigenetic activation of CTGF transcription. (a–e) Renal tubular epithelial cells were isolated from WT and KO mice as described in Methods. The cells were treated with TGF-β for 48 hr and ChIP/Re-ChIP assays were conducted with indicated antibodies. (f–j) Primary renal tubular epithelial cells were isolated from WT mice and treated with TGF-β in the presence or absence of CCG (10 μM) for 48 hr. ChIP/Re-ChIP assays were conducted with indicated antibodies. ChIP, chromatin immunoprecipitation; TGF-β, tumor necrosis factor-β

it was reasoned that CTGF might regulate MKL1 activity by impacting subcellular localization of MKL1. TGF-β treatment promoted the nuclear enrichment of MKL1 in NRK-52E cells; CTGF knockdown, however, severely disturbed the translocation of MKL1 to the nucleus (Figure 6e). We then transfected into NRK-52E a truncated form of MKL1 (MKL1 CA), which lacks the N-terminal RPEL domain and thus becomes constitutively nucleus-destined (Cen et al., 2003). As shown in Figure 6f, overexpression of MKL1 CA

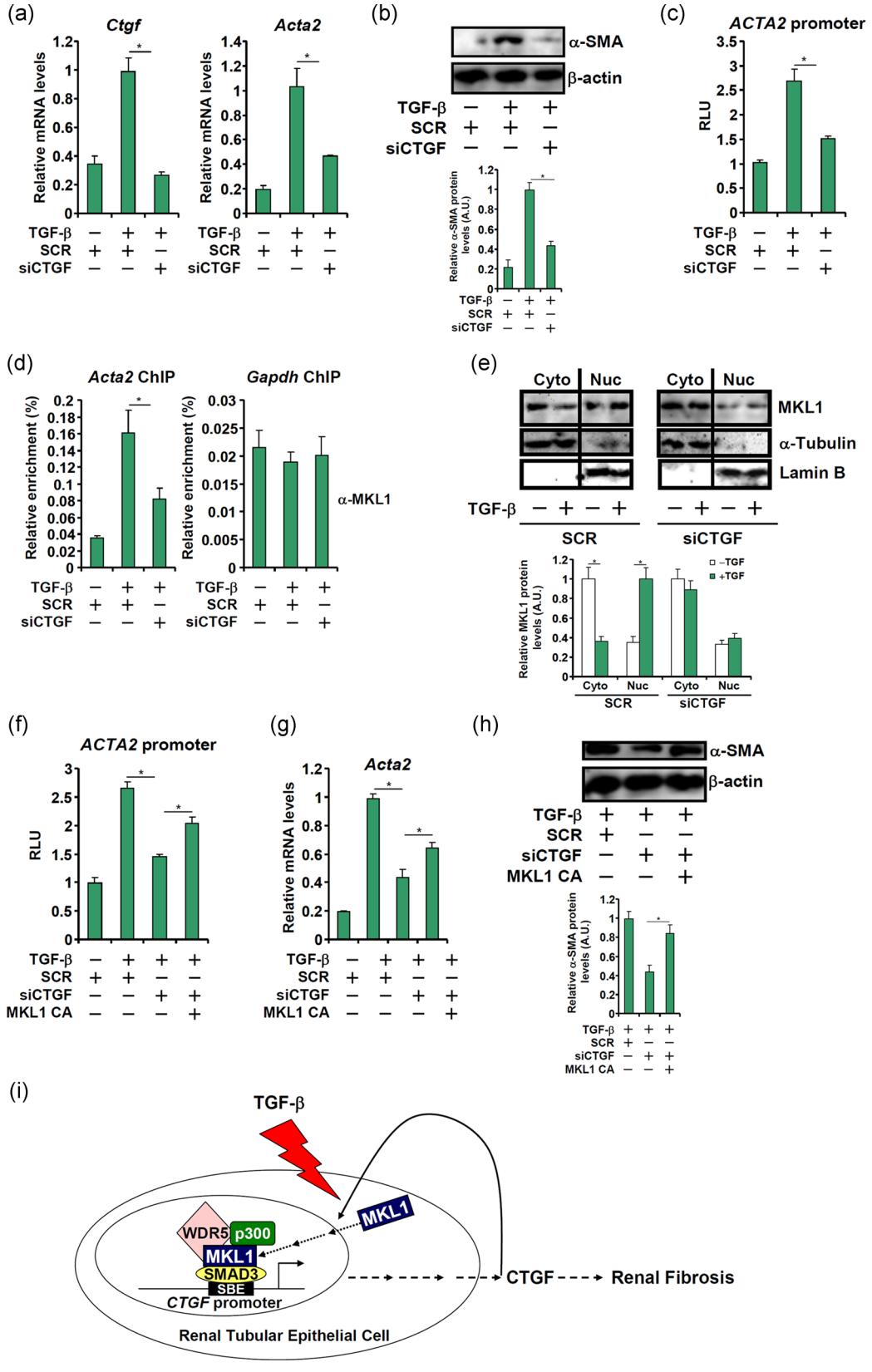

FIGURE 6 Continued.

largely bypassed the repressive effect of CTGF knockdown on the trans-activation of the Acta2 promoter. Furthermore, MKL1 CA partially restored the induction of endogenous α-SMA by TGF-β even in the absence of CTGF (Figure 6g,h). Combined, these data suggest that CTGF might potentially contribute to TGF-β induced pro-fibrogenic transcription in RTECs by regulating MKL1 activity.

4 | DISCUSSION

Rigorous mechanistic research and therefore exponential expansion of our understanding of its pathogenesis notwithstanding, renal fibrosis remains a daunting challenge for which effectual interventional solutions remain evasive. Here our data agree on a model in which the transcriptional modulator MKL1 regulates renal fibrosis in mice through epigenetic activation of the pro-fibrogenic cytokine CTGF in renal tubular epithelial cells (Figure 6i). First, it was demonstrated that genetic deletion of MKL1 attenuated UUO-induced renal fibrosis (Figure 1). This is consistent with our previous observation that MKL1 knockout mice were protected from diabetic nephropathy associated renal fibrosis
(Xu et al., 2015). Furthermore, it was shown that pharmaceutical inhibition of MKL1 by CCG-203971 also ameliorated UUO-induced renal fibrosis (Figure 2). This is in agreement with a recently published study showing that a structurally similar MKL1 inhibitor (CCG-1423) suppresses renal fibrosis in mice (Bialik et al., 2019). These data not only accord with previous reports showing the anti-fibrogenic effects of MKL1 inhibitors in peritoneal fibrosis (Sakai et al., 2013), scleroderma (Shiwen et al., 2015), and pulmonary fibrosis (Sisson et al., 2015) and solidify the pivotal role MKL1 plays in cellular fibrogenic response, but point to the potential of harnessing CCG family of compounds to treat renal fibrosis. Of note, neither MKL1 deletion (Figure 1a,b) nor its inhibition (Figure 2a,b) led to significant changes in glomerular filtration associated with ureteral tract obstruction. This is contradictory to previous findings in which MKL1 deficiency correlates with amelioration of glomerular function induced by septicemia (L. Liu et al., 2018), ischemia (L. Liu et al., 2018), or diabetes (Xu et al., 2015) in mice. This conflict suggests that MKL1 may regulate glomerular injury and renal fibrosis via separate mechanisms.

It can be said that MKL1-dependent transcriptional regulation often involves the epigenetic machinery. It was shown here that CTGF trans-activation is synonymous with p300-mediated histone H3 acetylation and WDR5-dependent histone H3K4 trimethylation surrounding the CTGF promoter (Figure 5). Administration of the nonspecific p300 inhibitor curcumin in mice protected against renal fibrosis in a broad range of different models (X. Sun et al., 2017), but it remains to be determined whether amelioration of renal fibrosis by curcumin treatment might be mediated or accompanied by normalization of renal CTGF levels. On the other hand, WDR5-null mice are unlikely to survive to term given its role in embryonic stem cell self-renewal (Ang et al., 2011). The unavailability of specific WDR5 inhibitors and appropriate genetic models preclude the analysis of its role in CTGF transcription and renal fibrosis in vivo. In addition, we suspect epigenetic regulation of CTGF transcription could be encapsulated by or reduced to H3 acetylation and H3K4 trimethylation. Natarajan and colleagues have previously shown that TGF-β induced CTGF transcription is partly mediated by the demethylase SET7/9 through modulating H3K9 trimethylation (G. Sun et al., 2010). The Jumonji domain-containing demethylase JMJD3 has also been implicated in TGF-β induced CTGF transcription by demethylating H3K27 (Estaras et al., 2012). A comprehensive epigenetic analysis of CTGF transcription in RTECs will unquestionably further our understanding of renal fibrosis pathogenesis and uncover novel targets that can be exploited to treat renal fibrosis.

Apparently, CTGF mediates TGF-β induced pro-fibrogenic transcription at least in part by promoting MKL1 nuclear translocation (Figure 6e). In support of this model, overexpression of a constitutively active MKL1 circumvented the requirement for CTGF to activate Acta2 transcription (Figure 6f,h). Yokoi et al. (2004) have found that depletion of CTGF with antisense oligos (ASO) attenuates renal fibrosis in mice. Interestingly, the anti-fibrogenic effect of CTGF depletion is achieved despite the presence of high levels of TGF-β in the kidneys. These observations combined seem to suggest that CTGF-dependent nuclear shutting of MKL1 might be the rate-limiting step in TGF-β induced fibrogenic response in RTECs. The mechanism whereby CTGF controls MKL1 translocation remains enigmatic at this point. It should be noted that CTGF has been shown to promote the trans-compartmental movement of other transcription factors including NF-κB (Sanchez-Lopez et al., 2009), Ets (P. S. Chen et al.,

FIGURE 6 CTGF is essential for TGF-β induced pro-fibrogenic transcription. (a and b) NRK-52E cells were transfected with small interfering RNA targeting CTGF or SCR followed by treatment with TGF-β. Quantitative PCR and western blot analysis were performed to determine gene expression levels. (c) A CTGF promoter-luciferase construct was transfected into NRK-52E cells with or without CTGF siRNA followed by treatment with TGF-β. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (d and e) NRK-52E cells were transfected with small interfering RNA targeting CTGF or SCR followed by treatment with TGF-β. ChIP assay was conducted with anti-MKL1. Subcellular localization was determined by western blot analysis. (f) A CTGF promoter-luciferase construct was transfected into NRK-52E cells with CTGF siRNA and constitutively active (CA) MKL1 followed by treatment with TGF-β. Luciferase activities were expressed as relative values compared with the control group arbitrarily set as 1. (g and h) NRK-52E cells were transfected with CTGF siRNA and constitutively active (CA) MKL1 followed by treatment with TGF-β. Quantitative PCR and western blot analysis were performed to determine gene expression levels. (i) A schematic model. ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; siRNA, small interfering RNA; TGF-β, tumor necrosis factor-β

2007), and β-catenin (Deng et al., 2007) alluding to a broad theme underlying CTGF-mediated pathophysiological processes.

In summary, our data demonstrate that MKL1 promotes renal fibrosis by activating CTGF transcription. CTGF, in turn, reciprocally regulates MKL1 activity by promoting its nuclear accumulation. Therefore, targeting the MKL1–CTGF axis may yield novel therapeutic solutions against aberrant renal fibrogenesis.

ACKNOWLEDGMENT

This study was supported by grants from the National Natural Science Foundation of China (81670618 and 81970638).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Y. X., X. Y. W., and T. Z. conceived the project; L. M., L. L., T. Y. Z., and X. Y. W. designed experiments, performed experiments, and collected data; Y. X. wrote the manuscript; X. Y. W. and T. Z. provided funding and supervision.

DATA AVAILABILITY STATEMENT

Research data not shared.

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How to cite this article: Mao L, Liu L, Zhang T, Wu X, Zhang T, Xu Y. MKL1 mediates TGF-β-induced CTGF transcription to promote renal fibrosis. *J Cell Physiol*. 2019;1–14. https://doi.org/10.1002/jcp.29356