The transcription factor Fosl1 preserves Klotho expression and protects from acute kidney injury

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Increased expression of AP-1 transcription factor components has been reported in acute kidney injury (AKI). However, the role of specific components, such as Fosl1, in tubular cells or AKI is unknown. Upstream regulator analysis of murine nephrotoxic AKI transcriptomics identified AP-1 as highly upregulated. Among AP-1 canonical components, Fosl1 was found to be upregulated in two transcriptomics datasets from nephrotoxic murine AKI induced by folic acid or cisplatin and from proximal tubular cells exposed to TWEAK, a cytokine mediator of AKI. Fosl1 was minimally expressed in the kidneys of control uninjured mice. Increased Fosl1 protein was localized to proximal tubular cell nuclei in AKI. In human AKI, FOSL1 was found present in proximal tubular cells in kidney sections and in urine along with increased urinary FOSL1 mRNA. Selective Fosl1 deficiency in proximal tubular cells (Fosl1Δtub) increased the severity of murine cisplatin- or folate-induced AKI as characterized by lower kidney function, more severe kidney inflammation and Klotho downregulation. Indeed, elevated AP-1 activity was observed after cisplatin-induced AKI in Fosl1Δtub mice compared to wild-type mice. More severe Klotho downregulation preceded more severe kidney dysfunction. The Klotho promoter was enriched in Fosl1 binding sites and Fosl1 bound to the Klotho promoter in cisplatin-AKI. In cultured proximal tubular cells, Fosl1 targeting increased the proinflammatory response and downregulated Klotho. *In vivo*, recombinant Klotho administration protected Fosl1Δtub mice from cisplatin-AKI. Thus, increased proximal tubular Fosl1 expression during AKI is an adaptive response, preserves Klotho, and limits the severity of tubular cell injury and AKI.

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Translational Statement

Fosl1 is 1 of 7 canonical components of the activator protein-1 (AP-1) transcription factor. While it is known that AP-1 is activated early in the course of acute kidney injury, the function of individual components is unknown. We have now uncovered evidence that supports that Fosl1 is increased in an adaptive manner in proximal tubular cells during acute kidney injury where it plays a kidney protective role, promoting the expression of the kidney protective protein Klotho and decreasing proinflammatory factors. These findings identify a kidney protective Fosl1-Klotho axis that may be exploited therapeutically in acute kidney injury and raises the spectrum of nephrotoxicity for cancer therapy targeting Fosl1.

Acute kidney injury (AKI) is characterized by a sudden decline of kidney function.[1] AKI increases the risk of chronic kidney disease, which is among the fastest-growing global causes of death.[2,3] Chronic kidney disease, in turn, predisposes to AKI. Treatment options for AKI are limited. Kidney function is replaced by dialysis when AKI is severe. Mortality in AKI may exceed 50%,[4] but the increased risk of death persists beyond a year.[5] An improved understanding of the molecular mechanisms of AKI will allow developing novel therapeutic approaches.

AKI is characterized by tubular cell injury leading to a proinflammatory stress response and cell dedifferentiation followed by tubular cell proliferation and recovery of kidney

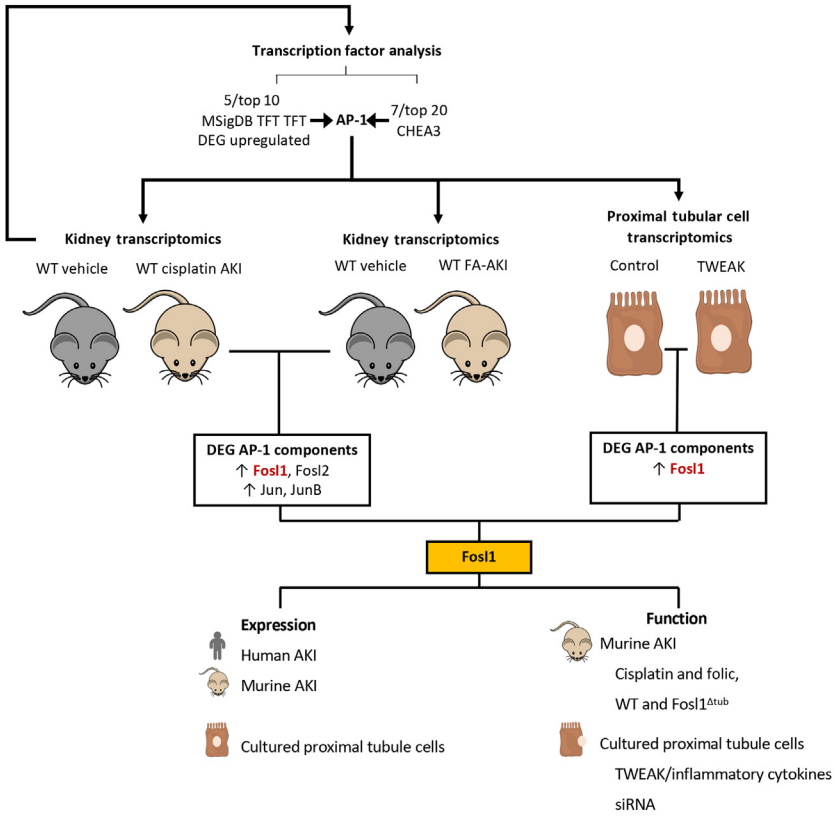

Figure 1 | Experimental design. Transcriptomics of murine kidneys with nephrotoxic acute kidney injury (AKI) and of murine proximal tubular cells exposed to tumor necrosis factor superfamily, member 12 (TWEAK) were used to identify key transcription factors upregulated in nephrotoxic AKI and to identify differentially expressed genes (DEGs) encoding components of key transcription factors relevant for both nephrotoxic AKI and proximal tubular cells. TWEAK is a key mediator of ischemia-reperfusion injury and nephrotoxic AKI. First, the activator protein-1 (AP-1) transcription factor was identified as upregulated in cisplatin-AKI (see Supplementary Tables S1 and S2). Then Fosl1 was identified as the only Fos family gene that was upregulated (i.e., fold-change >1.50, false discovery rate <0.05) during cisplatin- or folic acid (FA)–induced AKI and also by the cytokine TWEAK in cultured proximal tubule cells (see Supplementary Table S3). Thus, Fosl1 was chosen for detailed expression studies in murine and human kidneys and cultured murine tubular cells and for functional studies in mice with genetically modified proximal tubular cells (*Fosl1*$^{\Delta tub}$ mice) and in cultured proximal tubular cells following Fosl1 targeting by small, interfering RNA (siRNA). CHEA3, ChIP-X enrichment analysis 3; MSigDB, Molecular Signatures Database; TFT, transcription factor targets; WT, wild-type.

structure and function.[5] Inflammatory mediators such as tumor necrosis factor superfamily, member 12 (TWEAK) amplify kidney injury through recruitment of inflammatory cells, induction of regulated necrosis, and downregulation of kidney protective factors such as Klotho.[6–9] Loss of Klotho (Uniprot name), also termed α-Klotho, increases the severity of AKI and Klotho administration or prevention of Klotho downregulation through target gene activation protects from AKI.[6,10–13]

The transcription factor Fos-related antigen-1/Fos-like 1 (Fra1/Fosl1) is a leucine zipper protein from the FOS family (c-Fos, FosB, Fra1/Fosl1, Fra2/Fosl2).[14] Fosl1 is mainly activated by extracellular signal–regulated kinases 1 and 2 (ERK1/2) and p38 MAPK signaling[15] and heterodimerizes with JUN proteins (c-Jun, JunB, JunD) to form the canonical activator protein-1 (AP-1) transcription factor complex, which translocates to the nucleus and binds the consensus DNA sequence TGAC/GTCA.[16] Different AP-1 dimers display similar DNA binding specificities but differ in their transactivation potential.[17] Fosl1 lacks the C-terminal transactivation domain and is thought to behave as a weak activator and even as a repressor of transcription.[18]

The relative abundance of different combinations of Fosl1 and Jun family members depends on cell type and environment.[19] *Fosl1* is a constitutive gene essential for embryonic development[20,21] that regulates key cell functions that may be relevant for the pathogenesis of AKI, such as proliferation, differentiation, survival, epithelial-mesenchymal transition, inflammation, cell fate, and stem cell reprogramming.[22–26] Fosl1 is overexpressed in malignancy,[27,28] but may be also involved in inflammatory diseases.[29,30] It is a transcriptional regulator of neutrophil gelatinase-associated lipocalin in macrophages.[31] A very early increased gene expression of c-Jun and c-Fos in nephrotoxic and kidney ischemia-reperfusion injury (IRI) has been known for over 30 years.[32,33] However, the precise role of the AP-1 transcription factor and of individual components in AKI remained poorly understood.

We used kidney transcriptomics to identify novel players in the pathogenesis of AKI that may be targeted therapeutically. Specifically, we focused on highly expressed and active transcription factors and identified AP-1 as a highly active transcription factor and Fosl1 as having functions in tissue injury and repair and being highly expressed in AKI and in murine

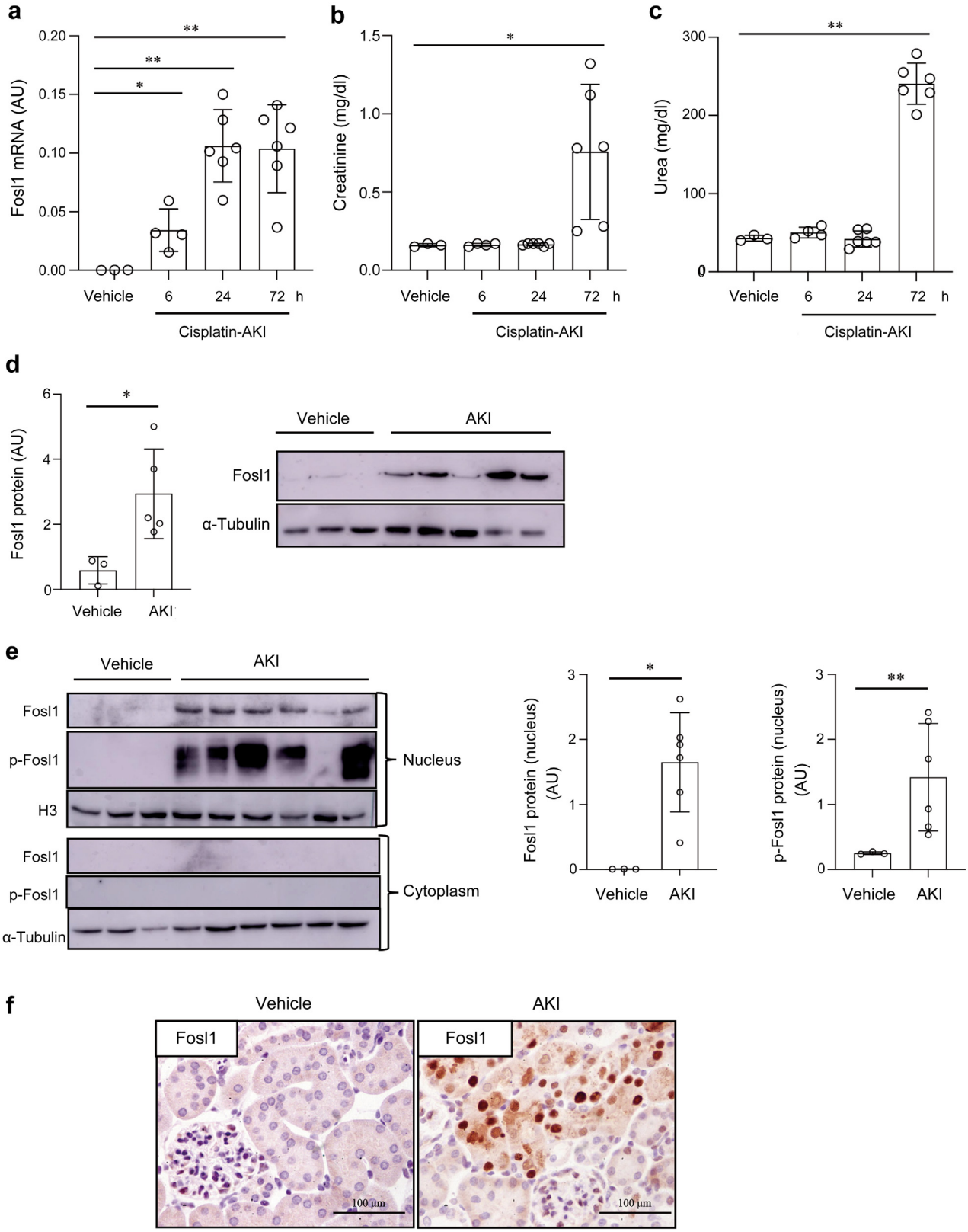

Figure 2 | Kidney Fosl1 expression is increased in murine nephrotoxic acute kidney injury (AKI) induced by cisplatin. (**a**) In cisplatin-induced AKI, *Fosl1* mRNA (quantitative reverse transcription polymerase chain reaction) was already upregulated at 6 hours and peaked at 24 to 72 hours, (**b,c**) preceding the increase in plasma creatinine (**b**) and urea (**c**). *$P < 0.001$ versus vehicle, **$P < 0.0001$ versus vehicle. Although Fosl1 mRNA was undetectable in whole control kidney, a more sensitive reverse transcription polymerase chain reaction approach detected minute amounts of Fosl1 mRNA under control conditions (see Supplementary Figure S5). (**d**) Kidney Fosl1 protein assessed by (continued)

proximal tubular cells exposed to a mediator of AKI. We now report that Fosl1 is part of an adaptive kidney protective response during AKI in proximal tubular cells that preserves Klotho and limits the inflammatory response and the severity of tubular cell injury and AKI.

METHODS

Figure 1 summarizes the experimental approach. Kidney and kidney cell transcriptomics identified key transcription factors upregulated in nephrotoxic AKI and differentially expressed genes encoding key transcription factors relevant for proximal tubular cells. The AP-1 transcription factor was upregulated in AKI (Supplementary Tables S1 and S2) and *Fosl1* was the only Fos family gene upregulated >1.5-fold by the cytokine TWEAK in cultured proximal tubule cells and during both cisplatin and folic acid (FA)–induced AKI (Supplementary Table S3). TWEAK is a key mediator of AKI.[6–9] Fosl1 was prioritized for detailed expression and functional studies. Extended Supplementary Methods provide further detail.

Animal models

C57BL/6 mice carrying the floxed *Fosl1* gene (*Fosl1*$^{f/f}$ mice, Erwin F. Wagner MGI:3055991 aka *Fosl1*^{tm2Wag})[33,34] were intercrossed with *PEPCK*^{Cre} transgenic mice expressing Cre recombinase under control of the mouse phosphoenolpyruvate carboxykinase (*Pepck*) promoter (Volker Haase, Vanderbilt University).[35,36] Male (cisplatin-AKI) or female (FA-AKI) 10- to 12-week-old mice were studied.[37–39,8,38,40,41] Some cisplatin-AKI mice were dosed with Klotho.

Kidney and kidney cell transcriptomics

A transcriptome analysis using Affymetrix arrays in kidney tissue 24 hours after FA or vehicle injection in wild-type (WT) C57BL/6 female mice and in cultured murine cortical tubule (MCT) proximal tubular cells after 6 hours of exposure to 100 ng/ml TWEAK or vehicle has been previously reported.[37,41] Transcriptome analysis by RNA-sequencing was performed in male C57BL/6 mice 24 hours after injection of cisplatin 20 mg/kg or vehicle.[42–45]

Cells

MCT murine proximal tubular cells were studied.[46–48]

Statistics

Statistical analysis was performed using GraphPad Prism Software 8. Results are expressed as mean ± SD. Significance ($P < 0.05$) was assessed by Student's *t*-test for 2 groups of data and analysis of variance for 3 or more groups with Bonferroni *post hoc* correction.

RESULTS

Fosl1 is an AP-1 component expressed by stressed proximal tubular cells of potential interest for AKI

Upstream regulator analysis of kidney transcriptomics from murine cisplatin-induced AKI identified AP-1 as being among the top upregulated transcription factors (Supplementary Table S1). Furthermore, transcription factor ChIP-X enrichment analysis 3 identified 7 of 20 top-ranked transcription factors as AP-1 heterodimer components (Supplementary Table S2). Of these, *Fosl1* and *Fosl2* mRNA were upregulated in both FA- and cisplatin-induced AKI, and *Fosl1* was additionally upregulated in proximal tubular cells exposed to TWEAK, a cytokine that contributes to nephrotoxic and IRI-AKI[9,49] (Supplementary Table S3). No *Jun* gene was upregulated simultaneously in cisplatin-AKI, FA-AKI, and TWEAK-MCT cells at false discovery rate <0.05 level. Thus, we focused on unraveling the regulation of the expression and activation as well as function of Fosl1 in proximal tubular cells and in AKI.

Increased Fosl1 expression in preclinical and clinical AKI

Reverse transcription quantitative polymerase chain reaction confirmed transcriptomics findings of increased kidney *Fosl1* mRNA expression in cisplatin-induced AKI (Figure 2a). *Fosl1* mRNA was already increased at 6 hours, preceding the increase in plasma creatinine and urea (Figure 2b and c), and peaked at 72 hours. Kidney Fosl1 protein levels (Figure 2d) and nuclear Fosl1 and phosphorylated Fosl1 (Figure 2e) were also increased in cisplatin-induced AKI consistent with Fosl1 activation.[50] Immunohistochemistry localized the increased expression of Fosl1 to tubular cell nuclei (Figure 2f) and nuclear Fosl1 also increased progressively from 6 hours (Supplementary Figure S1A). Specifically, Fosl1 was observed in tubular cell nuclei staining for the proliferation marker PCNA (Supplementary Figure S1B). Kidney c-Jun protein also increased during cisplatin-induced AKI and increased mRNA preceding increased protein levels (Supplementary Figure S2A and B). Reverse transcription quantitative polymerase chain reaction confirmed increased kidney *Fosl1* mRNA expression in FA-AKI (Supplementary Figure S3A) where Fosl1 also localized to tubular cell nuclei (Supplementary Figure S3B) and was associated to increased c-Jun (Supplementary Figure S2C and D).

Supporting the clinical translation of the findings, immunohistochemistry showed multiple FOSL1-positive tubular cells in human AKI but not in normal kidney tissue (Supplementary Figure S4A). In the urinary sediment of patients with AKI, *FOSL1* mRNA levels were up to 40-fold higher than in controls, and FOSL1 protein colocalized with proximal tubular cell markers while FOSL1-positive proximal tubular cells were not observed in control urine sediments (Supplementary Figure S4B and C).

Proximal tubular cell Fosl1 deficiency increases the severity of murine nephrotoxic AKI

To test the functional relevance of Fosl1 in kidney disease, we generated mice with proximal tubule–specific *Fosl1* deletion, in

Figure 2 | (continued) Western blot at 72 hours following injection of cisplatin or vehicle. *$P < 0.03$ versus vehicle. (**e**) Western blot analysis of kidney nuclear and cytoplasmic Fosl1 and phosphorylated (p-) Fosl1 protein at 72 hours following injection of cisplatin or vehicle. *$P < 0.008$, **$P < 0.05$ versus vehicle. Mean ± SD of 3 to 6 animals per group. (**f**) Immunohistochemistry localized Fosl1 expression to tubular cells at 72 hours in cisplatin-AKI. Original magnification ×40. Data expressed as mean ± SD of 4 to 6 animals per group. AU, arbitrary units; H3, histone 3. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

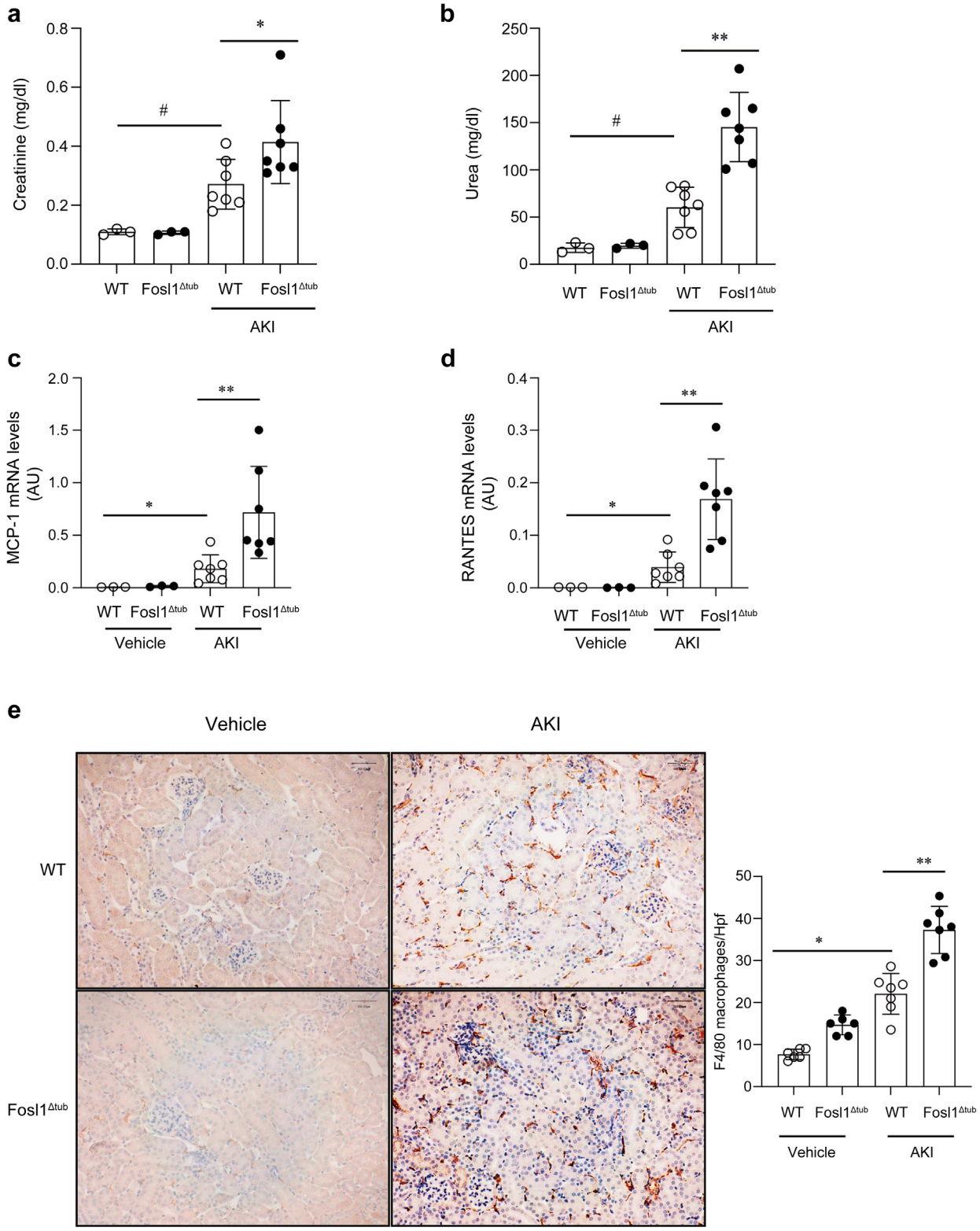

Figure 3 | Proximal tubular Fosl1 deficiency results in more severe cisplatin-induced acute kidney injury (AKI). (**a**) Plasma creatinine and (**b**) plasma urea: $^{*}P < 0.04$, $^{**}P < 0.0002$ versus wild-type (WT) mice with AKI, $^{\#}P < 0.02$ versus WT vehicle. (**c,d**) Kidney mRNA expression of genes encoding the chemokines monocyte chemoattractant protein-1 (MCP-1) (**c**) and regulated on activation, T-cell expressed, and secreted (RANTES) (**d**) is higher in mice with genetically modified proximal tubular cells (*Fosl1*$^{\Delta tub}$ mice) with AKI than in WT mice with AKI. Quantitative reverse transcription polymerase chain reaction: $^{*}P < 0.05$ versus vehicle, $^{**}P < 0.02$ versus WT AKI mice. (**e**) F4/80 macrophage immunohistochemistry. Macrophage infiltration is higher in *Fosl1*$^{\Delta tub}$-deficient mice with AKI than in WT mice with AKI. $^{*}P < 0.0001$ versus vehicle or WT AKI mice. Original magnification ×20. Time point for all studies: 72 hours. Data expressed as mean ± SD of 3 to 7 animals per group. Hpf, high-power field. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

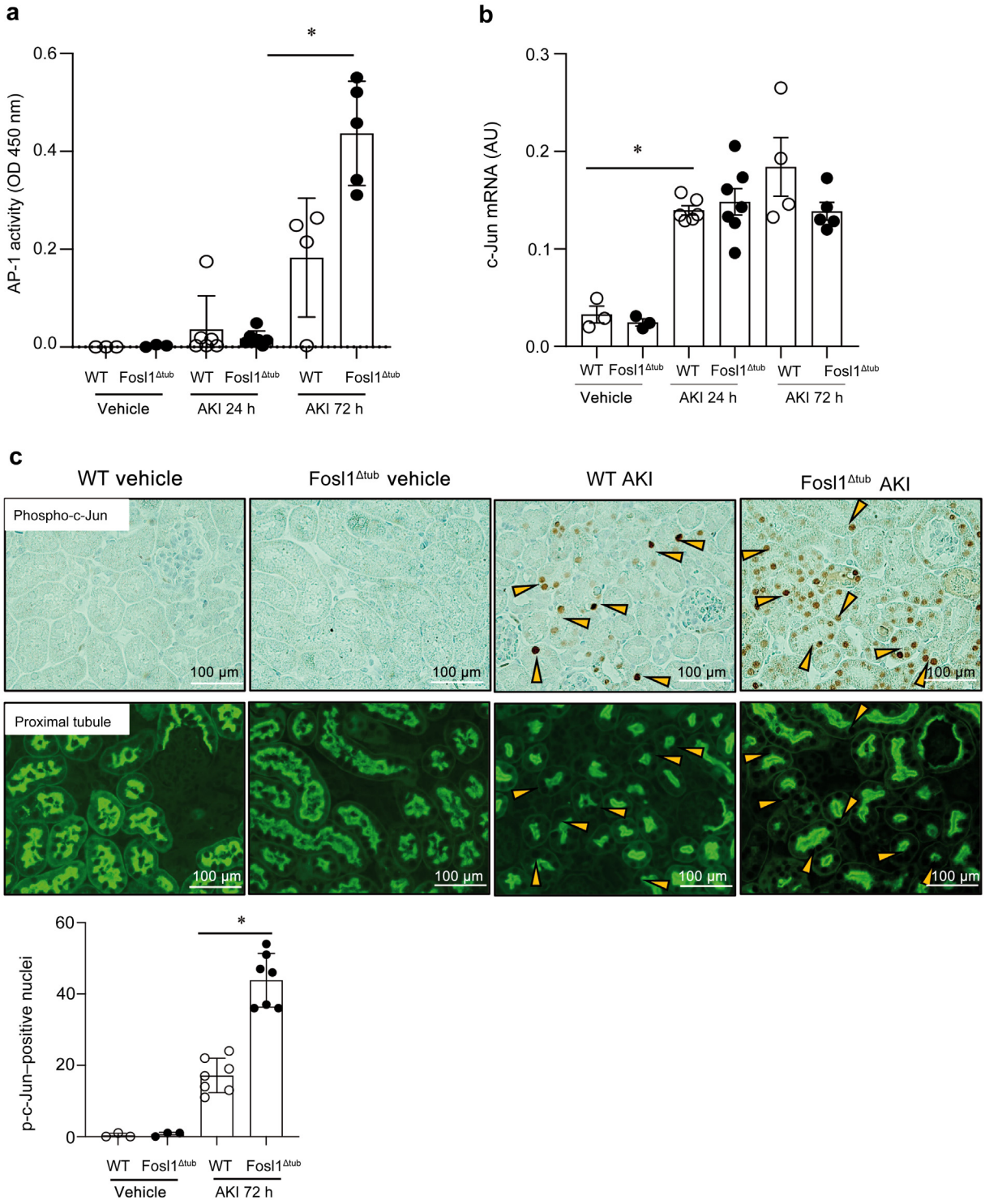

Figure 4 | Proximal tubular Fosl1 deficiency is associated with higher kidney transcriptional activator protein-1 (AP-1) activity for c-Jun during acute kidney injury (AKI). (**a**) Kidney nuclear extracts enzyme-linked immunosorbent assay for c-Jun AP-1 activity. *$P < 0.0001$ versus wild-type (WT) AKI at 72 hours. (**b**) Kidney c-Jun mRNA expression was not different in mice with genetically modified proximal tubular cells (*Fosl1*$^{\Delta tub}$)-deficient mice with AKI than in WT mice with AKI. *$P < 0.001$ versus vehicle. (**c**) Immunohistochemistry for p-Ser73-c-Jun costained with the proximal tubule marker lotus tetragonolobus lectin at 72 hours. Orange arrows point to p-Ser73–c-Jun–positive nuclei in proximal tubular cells identified by green lotus tetragonolobus lectin staining. Original magnification ×40. Data expressed as mean ± SD of 3 to 7 animals per group. OD, optical density. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

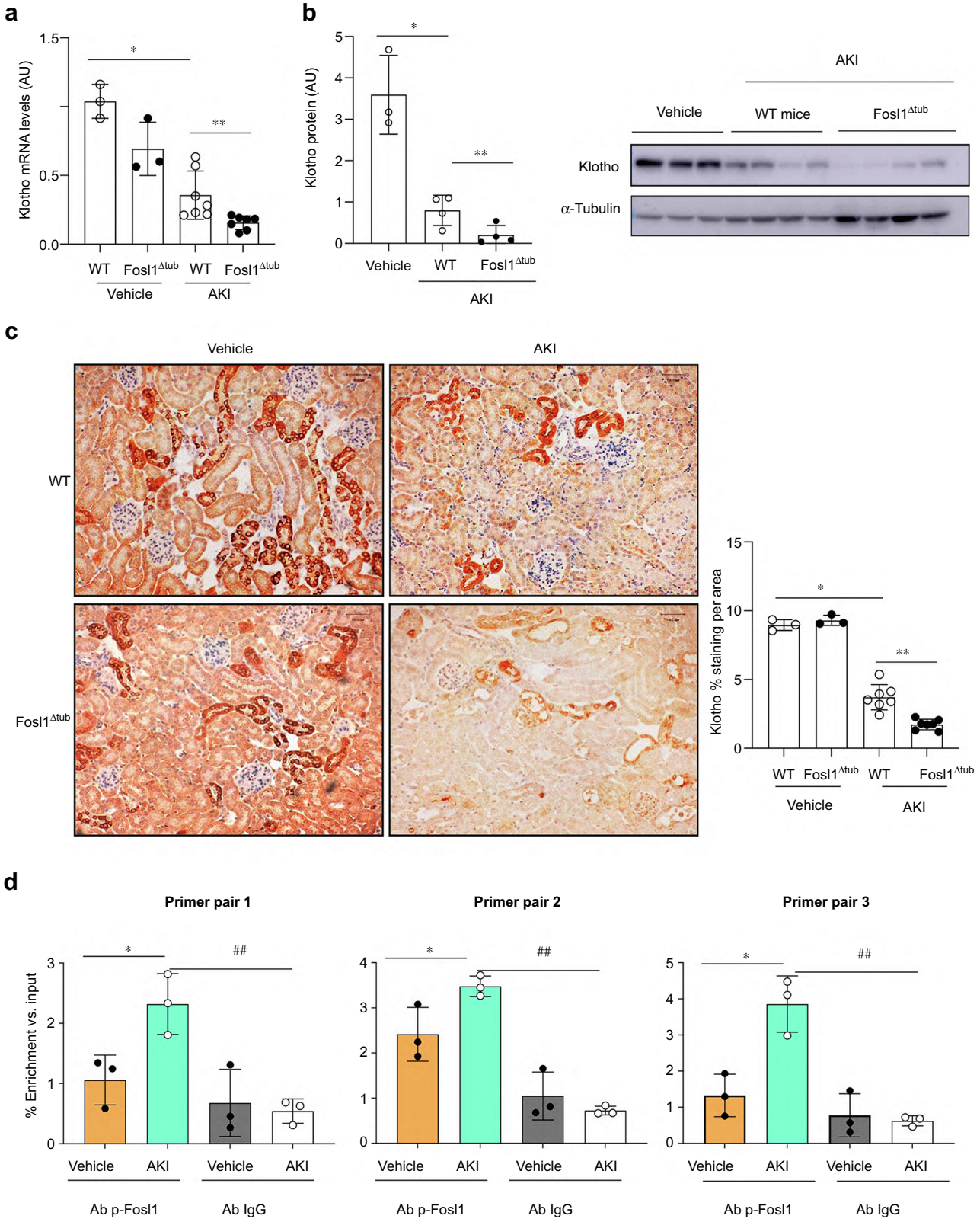

Figure 5 | Fosl1$^{\Delta tub}$ was associated with more severe Klotho downregulation in cisplatin-induced acute kidney injury (AKI). (**a,b**) Decreased whole kidney Klotho mRNA (**a**) and protein (**b**) expression in mice with genetically modified proximal tubular cells (*Fosl1*$^{\Delta tub}$ mice) with AKI compared to wild-type (WT) mice with AKI. *$P < 0.03$ versus vehicle, **$P < 0.03$ versus WT AKI. (**b**) Kidney Klotho. Representative Western blot and quantification. The main Klotho isoform in whole kidney is 130 kDa. (**c**) Immunohistochemistry showing more severe Klotho downregulation in *Fosl1*$^{\Delta tub}$ mice with AKI compared to WT mice with AKI. Smaller, more intensely stained tubules represent distal tubules, while proximal tubules present milder staining in WT vehicle control kidneys that is virtually lost in *Fosl1*$^{\Delta tub}$ mice with AKI. (continued)

which Fosl1 mRNA could not be amplified in healthy kidneys (Supplementary Figure S5). Fosl1 immunohistochemistry confirmed selective deletion of *Fosl1* in proximal tubules from *Fosl1*$^{f/f}$ *PEPCK*$^{Cre/-}$ (*Fosl1*$^{\Delta tub}$) mice, even during AKI, compared to multiple proximal tubular cells expressing Fosl1 in WT (*Fosl1*$^{f/f}$) mice with AKI (Supplementary Figure S6).

The overall baseline kidney histology of *Fosl1*$^{\Delta tub}$ mice was unremarkable and plasma creatinine and urea were normal. However, *Fosl1*$^{\Delta tub}$ mice developed a more severe cisplatin-induced AKI characterized by higher plasma creatinine and urea levels and more severe histological injury than in WT littermates with AKI at 72 hours (Figure 3a and b, Supplementary Figure S7A). Furthermore, *Fosl1*$^{\Delta tub}$ mice with AKI also displayed kidney expression of chemokine (monocyte chemoattractant protein-1; regulated on activation, T-cell expressed, and secreted [RANTES]) mRNA (Figure 3c and d), tubular cell tumor necrosis factor-α (TNF-α) immunostaining (Supplementary Figure S7A) and infiltration by interstitial macrophages (Figure 3e). These results suggest that Fosl1 upregulation in nephrotoxic AKI is an adaptive response and that precluding this adaptive response activates an amplification loop of inflammation and kidney injury.

Next, we examined whether proximal tubular *Fosl1* deficiency modulated AP-1 transcriptional activity *in vivo*. Confirming bioinformatics predictions, AKI was associated with increased whole kidney AP-1 transcriptional activity that was already observed at 24 hours and peaked at 72 hours. In *Fosl1*$^{\Delta tub}$ mice with AKI, peak AP-1 transcriptional activity at 72 hours was higher than in WT littermates with AKI (Figure 4a). While this increased AP-1 activity was not associated to increased c-Jun mRNA levels (Figure 4b), increased nuclear p-Ser73-c-Jun staining was observed at 72 hours (Figure 4c). Thus, proximal tubular cell Fosl1 deficiency did not result in an overall decrease in kidney AP-1 activity, rather it was associated with an increased AP-1 and c-Jun activity.

The impact of proximal tubular cell *Fosl1* deficiency was studied in murine nephrotoxic AKI induced by FA (72-hour time point). As for cisplatin-induced AKI, *Fosl1*$^{\Delta tub}$ mice had more severe FA-AKI as assessed by plasma creatinine or urea (Supplementary Figure S3C and D).

Fosl1 deficiency results in suppressed Klotho expression

To provide insight into the drivers of the more severe AKI in *Fosl1*$^{\Delta tub}$ mice, we generated kidney transcriptomics profiles of *Fosl1*$^{\Delta tub}$ and WT mice with cisplatin AKI. RNA-sequencing revealed 146 differentially expressed genes ($P < 0.05$), 73 upregulated and 73 downregulated, in *Fosl1*$^{\Delta tub}$ mice with AKI when compared to WT littermates with AKI (Supplementary Table S4, Supplementary Figure S8A). Principal component analysis clearly separated *Fosl1*$^{\Delta tub}$ AKI and WT AKI mice (Supplementary Figure S8B). The top-10 downregulated genes, all fold-change <0.50, included *Kl* encoding Klotho (fold-change 0.48) and *Cyp24a1* (fold-change 0.28) encoding the 25-hydroxy-vitamin-D-24-hydroxylase, an enzyme downstream of FGF23/Klotho signaling in proximal tubular cells whose expression is known to decrease under conditions of Klotho deficiency (Supplementary Figure S8C). None of the other top-10 downregulated genes has a known function in AKI (according to a PubMed search on August 31, 2022). Upregulated genes included c-Fos and FosB, potentially contributing to higher AP-1 activity.

AKI is characterized by decreased expression of the anti-inflammatory, nephroprotective, and antiaging factor Klotho, and preventing Klotho downregulation prevents AKI.[11–13] Thus, we further characterized the interaction between Fosl1 and Klotho. The more severe downregulation of kidney Klotho mRNA (Figure 5a) and protein (Figure 5b and c) in *Fosl1*$^{\Delta tub}$ mice with AKI than in WT mice with AKI was confirmed by reverse transcription polymerase chain reaction, Western blot, and immunohistochemistry, suggesting a potential contribution of endogenous Fosl1 to preserve Klotho expression. Indeed, Klotho downregulation in *Fosl1*$^{\Delta tub}$ mice was severe in proximal tubular cells (Figure 5c). Additionally, the lower Klotho expression in *Fosl1*$^{\Delta tub}$ mice with AKI than in WT mice with AKI was already observed at 24 hours (Supplementary Figure S9D), thus preceding other evidence of kidney injury such as higher plasma creatinine (Supplementary Figure S9A) and higher chemokine expression (Supplementary Figure S9B and C). In line with the lower Klotho expression, FGF23 and phosphate levels started to increase already at 24 hours in some *Fosl1*$^{\Delta tub}$ mice with AKI and were higher at 72 hours in *Fosl1*$^{\Delta tub}$ mice with AKI than in WT AKI mice (Supplementary Figure S10A and B). Moreover, enrichment of Fosl1 binding sites was found in the Klotho promoter (Supplementary Figure S11) and chromatin immunoprecipitation assays confirmed direct Fosl1 binding to the Klotho promoter in the kidney during cisplatin-AKI (Figure 5d). These results suggest that Klotho expression is regulated through Fosl1 in cisplatin-AKI and Fosl1 deficiency results in lower Klotho levels.

Figure 5 | (continued) Representative image. Original magnification ×20. *$P < 0.0001$ versus vehicle, **$P < 0.001$ versus WT AKI. Data expressed as mean ± SD of 3 to 7 animals per group at 72 hours. (**d**) Fosl1 binds to the Klotho promoter during AKI. Chromatin was immunoprecipitated with an anti-phosphorylated (p)-Fosl1 antibody (Ab) or with IgG and putative Klotho promoter binding sites for Fosl1 were amplified by quantitative polymerase chain reaction in duplicate using specific primers. Normal rabbit IgG was used as negative control for the specificity of the immunoprecipitation. As a positive control, aliquots of chromatin fragments obtained before immunoprecipitation were also subjected to reverse transcription polymerase chain reaction analysis (input). Immunoprecipitated DNA with p-Fosl1 binding was normalized to input chromatin. Data are expressed as fold enrichment of p-Fosl1 binding compared with negative control antibody (normal rabbit IgG). n = 3. *$P < 0.05$ versus vehicle Ab p-Fosl1, ##$P < 0.005$ versus AKI IgG. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

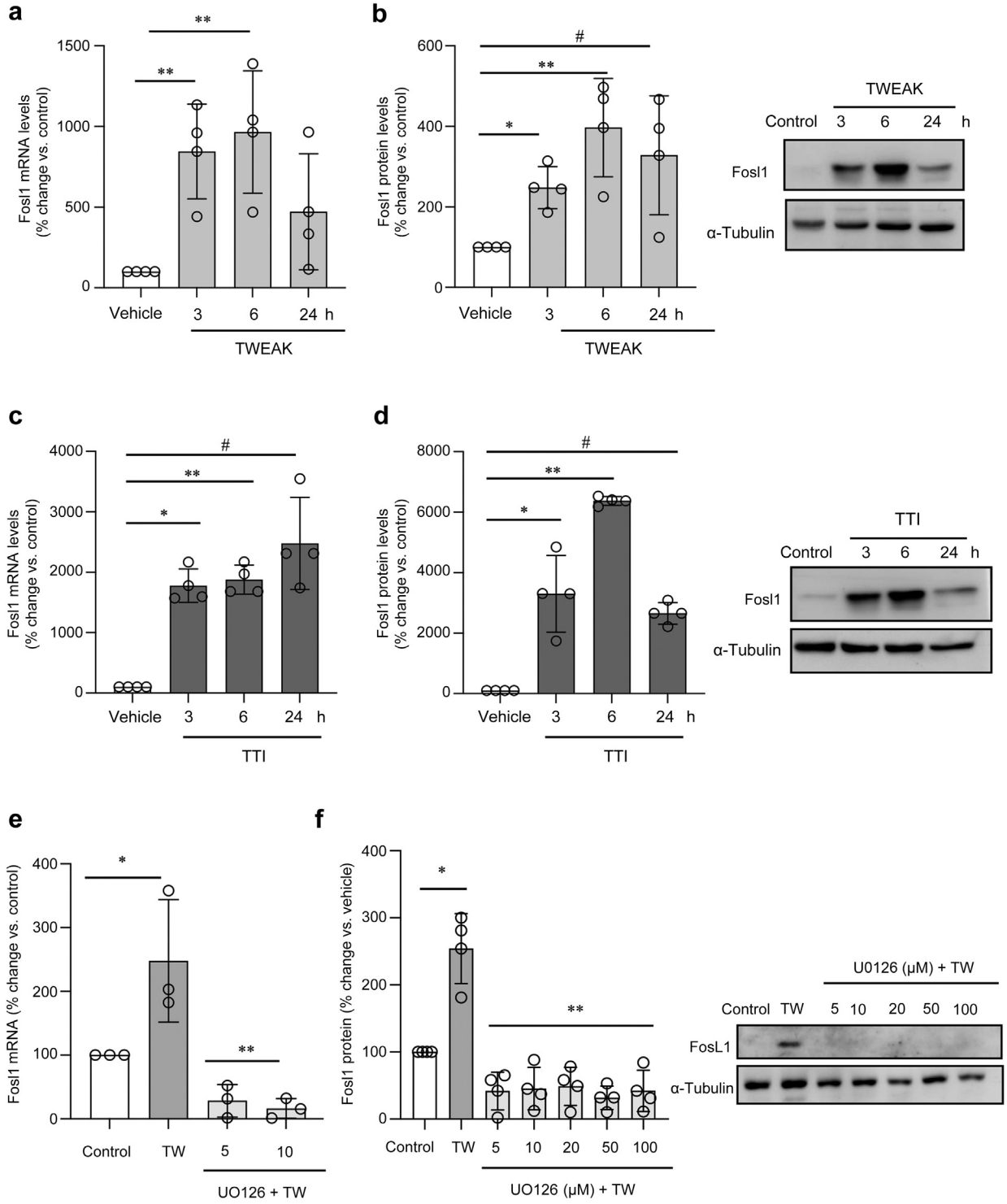

Figure 6| Inflammatory cytokines and MAPK kinase-1 (MEK1)/MEK2 upregulate Fosl1 expression in cultured tubular cells. Murine proximal tubular murine cortical tubule (MCT) cells were exposed to 100 ng/ml tumor necrosis factor superfamily, member 12 (TWEAK) or to TWEAK/tumor necrosis factor-α/interferon-γ (TTI). (**a**) Fosl1 mRNA and (**b**) protein expression increased in response to TWEAK. (**c**) Fosl1 mRNA and (**d**) protein expression also increased in response to TTI. Quantitative reverse transcription polymerase chain reaction. $^{*}P < 0.001$ versus vehicle, $^{**}P < 0.03$ versus vehicle, $^{\#}P < 0.02$ versus vehicle. (**e,f**) The MEK1/MEK2 inhibitor U0126 dose-dependently inhibits TWEAK-induced Fosl1 expression in MCT cells as assessed by quantitative reverse transcription polymerase chain reaction (**e**) for mRNA ($^{*}P < 0.02$ vs. vehicle, $^{**}P < 0.002$ vs. TWEAK [TW] alone) and by Western blot (**f**) for protein. Representative Western blot and quantification. $^{*}P < 0.0001$ versus vehicle, $^{**}P < 0.0001$ versus TWEAK alone. Mean ± SD of 4 independent experiments.

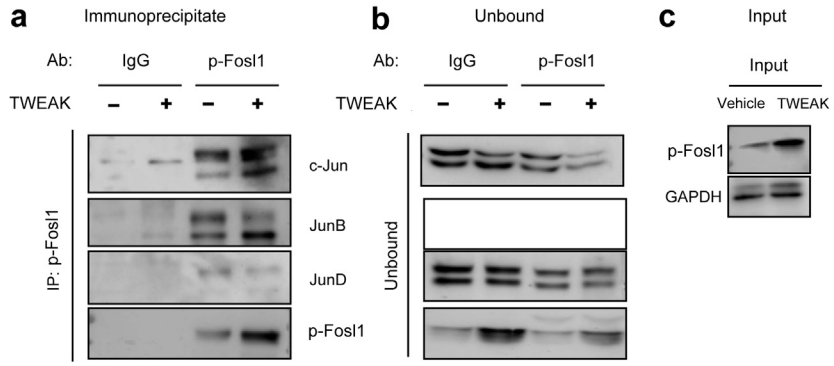

Figure 7 | Fosl1 differentially interacts with Jun family members in cultured proximal tubular cells in a proinflammatory milieu. Murine cortical tubule cell extracts following a 6-hour stimulation with tumor necrosis factor superfamily, member 12 (TWEAK) were immunoprecipitated (IP) with anti-phosphorylated (p)-Fosl1 antibody (Ab) or a nonspecific immunoglobulin (IgG). Western blots were probed for Fosl1 and Jun family proteins. (**a**) Immunoprecipitate. (**b**) Unbound. (**c**) Input. On TWEAK stimulation, Fosl1 recruited and bound c-Jun, as c-Jun is increased in the immunoprecipitated blot (**a**) but it decreased in the unbound blot (**b**). JunB is increased by TWEAK and similarly, Fosl1 clearly coimmunoprecipitates with JunB (**a**), decreasing the amount of protein in the unbound blot (**b**). However, Fosl1 barely interacts with JunD in these conditions. Representative Western blot of 3 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Further insight into the role of Klotho downregulation in increasing the severity of AKI in *Fosl1*$^{\Delta tub}$ mice was obtained by the systemic administration of recombinant Klotho, which decreased the severity of cisplatin AKI in *Fosl1*$^{\Delta tub}$ mice (Supplementary Figure S12).

Inflammatory cytokines regulate Fosl1 expression in cultured kidney tubular cells

Because baseline Fosl1 expression is very low in cultured or *in vivo* tubular cells, and during experimental AKI kidney Fosl1 expression is increased and localized to tubular cells, we explored drivers of increased Fosl1 expression and the function of Fosl1 in the cell response to these drivers.

TWEAK is a key mediator of AKI that in cultured tubular cells reproduces some of the gene expression changes observed in AKI, such as increased proinflammatory factor expression and decreased Klotho expression.[6,41,51,52] We explored proinflammatory stimuli represented by TWEAK and the combination of TWEAK with the proinflammatory cytokines TNF-α and interferon-γ (TTI). In cultured murine tubular cells, both TWEAK and TTI induced an early increase in *Fosl1* expression at the mRNA and protein levels (Figure 6a–d). *Fosl1* transcription is regulated by the MAPK kinases 1 and 2 (MEK1/2)/ERK1/2 pathway.[50] Indeed, the MEK1/2 inhibitor UO126 prevented ERK1/2 activation in response to TWEAK (Supplementary Figure S13A) and the TWEAK-induced upregulation of Fosl1 protein and mRNA in cultured tubular cells (Figure 6e and f). However, UO126 also prevented the proinflammatory impact of TWEAK on chemokine mRNA expression (Supplementary Figure S13B and C) and Klotho downregulation (Supplementary Figure S13D) consistent with the requirement of ERK1/2 to elicit these responses. This suggests that inflammation and Klotho downregulation (which promote kidney injury) and Fosl1 upregulation (kidney protective) share intracellular regulatory signaling.

The interaction of AP-1 subunits was analyzed by immunoprecipitation in cultured MCT cells stimulated with TWEAK. In line with transcriptomics data (Supplementary Table S3), TWEAK increased both phosphorylated Fosl1 and JunB protein (Figure 7). TWEAK stimulation resulted in AP-1 dimers containing phosphorylated Fosl1 (the active form of Fosl1) and either c-Jun or JunB, but barely JunD (Figure 7). These data identified c-Jun or JunB as preferential partners of Fosl1 in an inflammatory environment in proximal tubular cells that is consistent with kidney and cultured tubular cell transcriptomics data. Finally, we examined the effect of Fosl1 overexpression in TWEAK-stimulated cells. Fosl1 overexpression resulted in a milder TWEAK-induced increase in AP-1 transcriptional activity (Supplementary Figure S14), suggesting that Fosl1 prevents AP-1 overactivity.

Fosl1 function in cultured proximal tubular cells

The function of Fosl1 was explored in cultured proximal tubular epithelial MCT cells by small, interfering RNA (siRNA) targeting of Fosl1. Culture conditions for siRNA resulted in increased Fosl1 levels in MCT cells and a specific siRNA decreased Fosl1 at 48 and 72 hours (Figure 8a). Fosl1 silencing resulted in a spontaneous mild proinflammatory response characterized by increased monocyte chemoattractant protein-1 mRNA (Figure 8c and d) but unchanged RANTES mRNA (Supplementary Figure S15A and B). By contrast, Fosl1 silencing severely downregulated Klotho mRNA and protein expression, similar to cytokine stimulation, and further decreased Klotho expression in cytokine-stimulated cells (Figure 8e and f). Fosl1 siRNA also prevented Fosl1 upregulation induced by TWEAK (Figure 8b). Fosl1 silencing in TWEAK-stimulated tubular cells magnified monocyte chemoattractant protein-1 and RANTES mRNA overexpression in response to TWEAK and to the more potent stimulus TTI (Figure 8c and d, Supplementary Figure S15A and B). Moreover, in proximal tubular cells, Fosl1 silencing magnified the expression of TNF-α (Supplementary Figure S15C and D), a known suppressor of Klotho expression that may reach distal tubule cells. Overall,

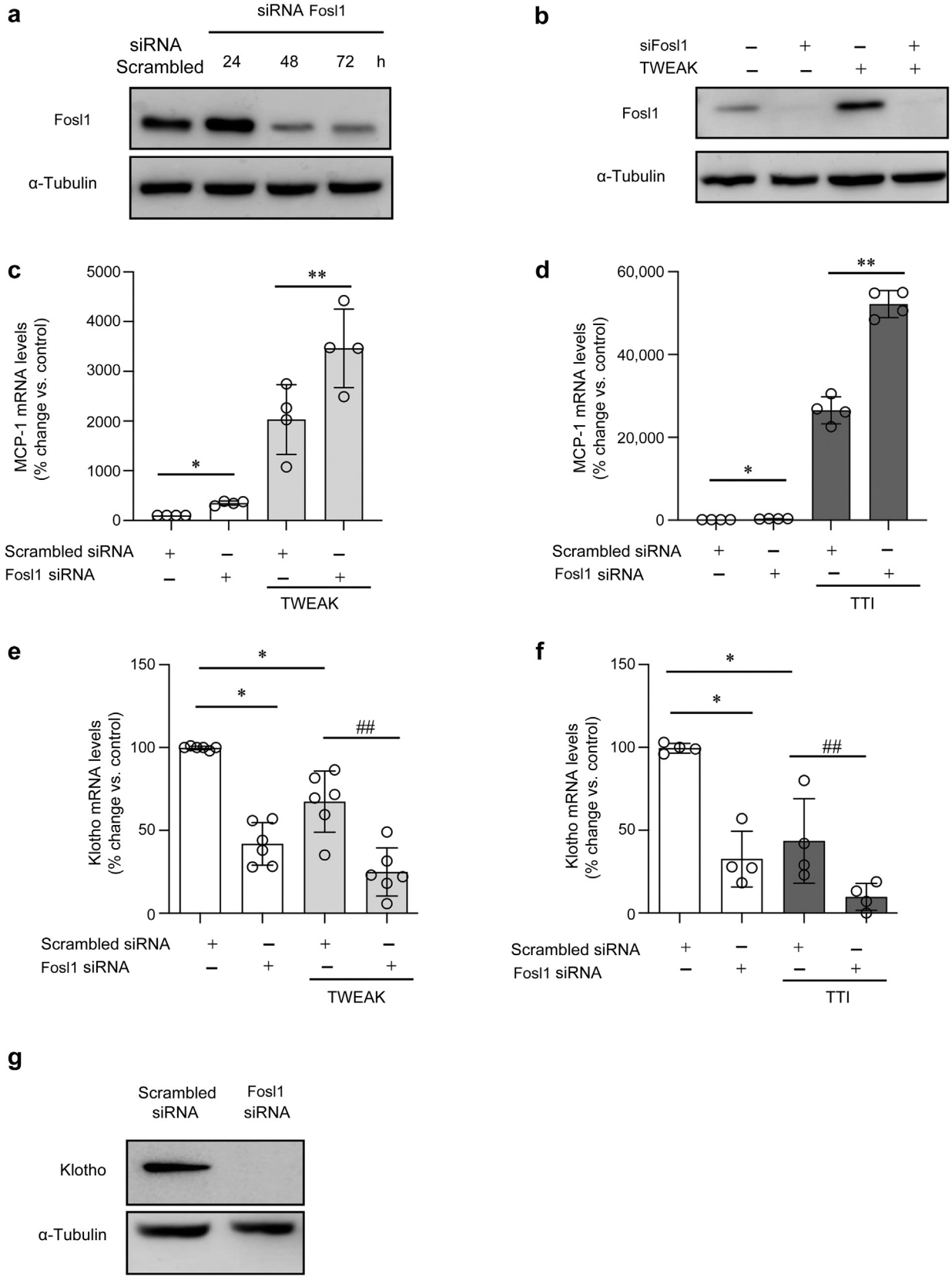

Figure 8 | Functional characterization of Fosl1 actions on cultured proximal tubular cells. (**a**) Culture conditions for small, interfering RNA (siRNA) targeting increased baseline Fosl1 expression in cultured murine proximal tubular cells and Fosl1 siRNA silencing suppressed Fosl1 protein expression. Representative Western blot. (**b**) Fosl1 siRNA silencing also suppressed tumor necrosis factor superfamily, member 12 (continued)

these data suggest that Fosl1 expression under conditions of cell stress induced by the silencing procedure or by proinflammatory cytokines limits adverse cell responses to stress such as Klotho downregulation or the proinflammatory response.

DISCUSSION

The main finding of this study is that following an insult, Fosl1 is rapidly upregulated in proximal tubular cells during AKI where it plays an adaptive kidney-protective role by dampening proinflammatory responses and promoting nephroprotective responses such as preservation of Klotho expression. This response was observed in 2 independent models of nephrotoxic AKI and in cultured tubular cells exposed to inflammatory stimuli that are known to contribute to the pathogenesis of AKI. Supporting a direct impact of Fosl1 on the regulation of Klotho expression, we characterized for the first time Fosl1 binding to the Klotho promoter and regulation of Klotho expression.

Fosl1 is 1 of the 4 members of the FOS family (c-Fos, FosB, Fosl1, Fosl) that together with JUN proteins form the canonical AP-1 transcription factor.[14] In the cytoplasm, Fosl1 activity is modulated by posttranslational modifications such as phosphorylation of serine residues S265 and S252.[28] Fosl1 requires heterodimerization with JUN proteins (c-Jun, JunB, and JunD) to form AP-1.[16] The different AP-1 dimers display similar DNA binding specificities but differ in their transactivation potential.[17] Contrary to c-Fos and FosB, Fosl1 lacks the C-terminal transactivation domain and thus is unable to transform established rodent fibroblast cell lines.[18] In this regard, unlike c-Fos overexpression, which causes osteosarcomas, Fosl1 overexpression caused a progressive increase in bone mass leading to osteosclerosis associated with increased osteoblast differentiation, but not proliferation, and also with enhanced osteoclastogenesis.[53] This inhibitory role of Fosl1 on AP-1 may have contributed to the increased AP-1 c-Jun activity observed *in vivo* in mice with AKI and proximal tubular cell Fosl1 deletion. Fosl1 is upregulated in malignancy[27,28] and has also been associated with arthritis,[29,30] psoriasis,[54,55] lipodystrophy,[56] and lung disorders.[57,58] Fosl1 is crucially involved in human tumor progression and metastasis and represents a promising therapeutic target.[19] An integrative analysis of 324 human cell lines (30 cancer types, 19 tissues)[59] identified Fosl1 as the AP-1 component that most favored cancer growth. However, no specific therapeutic strategy targeting Fosl1 is yet undergoing clinical trials. Given the present results, the clinical development of any such strategy should carefully evaluate the potential to increase the incidence and/or severity of nephrotoxicity.

c-Jun and c-Fos gene expression increases very early in nephrotoxic and IRI kidney injury.[32,33] However, the precise role of AP-1 and its individual components in AKI remained poorly understood. Recently, T-5224 was reported to inhibit the production of TNF-α and improve kidney function in lipopolysaccharide-injected mice.[60,61] T-5224 is a small molecule that inhibits c-Fos/AP-1 in promoter-luciferase assays, without affecting the levels of c-Fos family protein members and selectively inhibits the DNA binding activity of c-Fos/c-Jun. Contrary to the potential contribution of c-Fos/AP-1 to lipopolysaccharide-induced AKI, our data support a kidney protective role of Fosl1. Fosl1 is upregulated very early during proximal tubular cell injury in culture and *in vivo* during nephrotoxic AKI and specific deficiency of Fosl1 in proximal tubular cell results in more severe AKI characterized by worse kidney function, more severe kidney inflammatory cell infiltration, and more severe suppression of Klotho. Indeed, Fosl1 targeting increased the secretion of proinflammatory cytokines, such as TNF, that are known to decrease Klotho in distal tubular cells that may be exposed to proximal tubular cell TNF from the luminal or basolateral side.[62] The loss of tubular cell Klotho expression is an early event in AKI and chronic kidney disease, while preservation of Klotho expression or Klotho administration protects against AKI.[6,10–13] In this regard, Klotho has antiaging, antioxidant, anti-inflammatory, and antifibrotic properties.[41,63] A key role for Klotho suppression in the more severe kidney injury observed in mice with proximal tubular Fosl1 deficiency is supported by the finding of Fosl1 binding sites in the Klotho promoter, evidence of Fosl1 binding to the Klotho promoter during AKI, the early severe decrease in kidney Klotho *in vivo* that preceded other evidence of more severe kidney injury such as expression of inflammatory mediators or decreased kidney function, and the finding of Klotho and a downstream gene among the most downregulated genes when comparing Fosl1-deficient mice with WT mice with AKI. In cultured tubular cells, TWEAK activated MEK1/2/ERK1/2 to induce both a deleterious response (expression of inflammatory genes, downregulation of Klotho) and the expression of Fosl1, which functional studies revealed to limit the deleterious response. Both responses were abrogated by a MEK1/2 inhibitor, suggesting that TWEAK, even under the

Figure 8 | (continued) (TWEAK)–induced Fosl1 expression. (**c**) Fosl1 siRNA silencing magnifies the monocyte chemoattractant protein-1 (MCP-1) mRNA upregulation induced by a 6-hour stimulation with TWEAK (100 ng/ml). (**d**) Additionally, Fosl1 siRNA silencing also magnified the increase in MCP-1 mRNA expression induced by the more potent stimulus resulting from the combination of TWEAK with the proinflammatory cytokines tumor necrosis factor-α and interferon-γ I (TTI). In both cases, Fosl1 siRNA targeting also increased the baseline expression of MCP-1. $^{*}P < 0.0001$ versus scrambled siRNA, $^{**}P < 0.002$ versus scrambled siRNA + TWEAK or TTI. Real-time reverse transcription polymerase chain reaction. (**e–g**) In Fosl1 siRNA silenced cells, Klotho expression was spontaneously downregulated at the mRNA (**e,f**) (quantitative reverse transcription polymerase chain reaction) and protein (**g**) levels (representative Western blot). The main Klotho isoform in cultured tubular cells is 70 kDa. Additionally, Fosl1 siRNA resulted in a further decrease in Klotho expression as compared with cytokine stimulation alone (**e,f**). As for MCP-1, Fosl1 targeting also further suppressed Klotho expression on top of the more potent stimulus TTI. $^{*}P < 0.02$ versus scrambled siRNA, $^{\#\#}P < 0.05$ versus TWEAK or TTI. Mean ± SD of 4 independent experiments. Experiments were collected 48 hours after silencing.

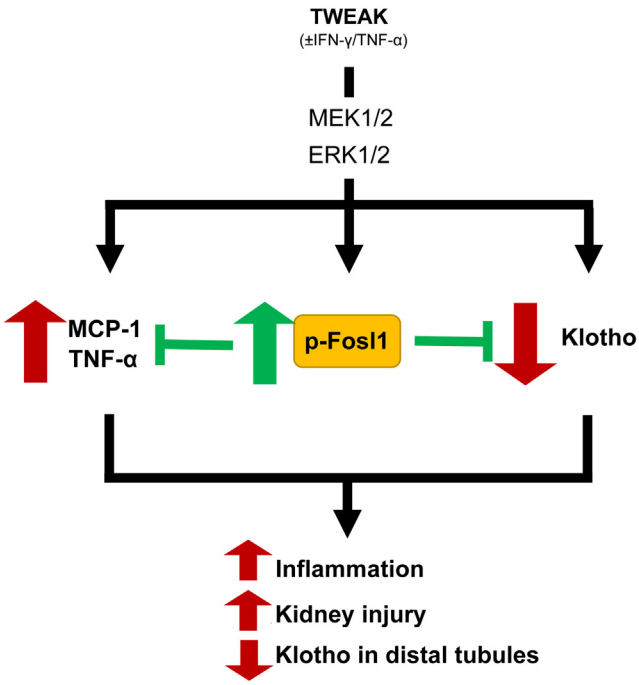

Figure 9 | Role of Fosl1 in acute kidney injury (AKI). In AKI, stressors such as tumor necrosis factor superfamily, member 12 (TWEAK) and, potentially, other stimuli, increase Fosl1 mRNA, protein, phosphorylation (p), and nuclear translocation. Preventing Fosl1 recruitment increased the severity of kidney injury *in vivo*, suggesting that Fosl1 upregulation represents a compensatory mechanism. In cultured tubular cells, TWEAK triggers Fosl1 upregulation, an inflammatory response (represented here by monocyte chemoattractant protein-1 [MCP-1] and tumor necrosis factor-α [TNF-α]) and Klotho downregulation through MAPK kinases 1 and 2 (MEK1/2) and extracellular signal–regulated kinases 1 and 2 (ERK1/2) signaling. Fosl1 prevents TWEAK-induced upregulation of proinflammatory mediators and downregulation of the kidney protective factor Klotho. Klotho appears to be a key direct target of the nephroprotective action of Fosl1 as binding of Fosl1 to the Klotho promoter was observed *in vivo* during AKI. It had been previously shown that Klotho administration, overexpression, or prevention of Klotho downregulation is nephroprotective in AKI. Additionally, Fosl1 decreased the expression of proinflammatory cytokines, such as TNF-α, known to decrease Klotho expression. Proximal tubular expression of TNF-α may downregulate Klotho expression in distal tubular cells, which can be exposed to TNF-α from proximal tubular cells either through the interstitium (adjacent distal tubular cells) or the tubular lumen (downstream distal tubular cells). IFN-γ, interferon-γ.

most unfavorable conditions such as the presence of additional mediators of inflammation (TTI) activates both a deleterious response and its corresponding brake, as evidenced by the consequences of Fosl1 silencing.

Proximal tubular cell Fosl1 deficiency was associated with an overall increase in kidney AP-1 activity in AKI. This may reflect more severe kidney disease and/or the involvement of other AP-1 partners, including c-Fos and FosB that were upregulated in AKI in Fosl1-deficient mice and/or availability of free c-Jun, c-Jun/c-Jun homodimers that are proinflammatory in enterocytes and fibroblasts cells.[64,65]

While there are no prior data on the function of Fosl1 during kidney injury, several recent studies have provided observational evidence that supports a key role of Fosl1 in AKI, as characterized in the present study. Thus, Fosl1 was the top transcription factor motif associated with increased enhancer elements in murine IRI AKI.[66] Also, in murine IRI-AKI, a single cell transcriptomics analysis found Fosl1 significantly upregulated in injured S3 cells while the Fosl1 regulon was upregulated in injured S1/S2 and S3 proximal tubular cells to a greater extent than in severely injured or failed repair proximal tubular cells, unlike JunB, JunD, and c-Jun or Fosl2 that clearly peaked in severely injured cells.[67] These observational data may be considered in line with our functional data, as upregulation of Fosl1 in injured but not in severely injured cells is consistent with Fosl1 driving an adaptive response that limits further injury, as observed in our studies. As for FA-AKI, in IRI-AKI, the Fosl1 upregulation in proximal tubules was transient (peaking at 4 hours, already decreasing by 12 hours, return to near baseline levels by 48 hours).[67]

Previous information on Fosl1 and inflammation outside the kidney context is derived from transgenic mice with enforced overexpressing Fosl1 or Fosl1/JunB, which does not provide information on the role of endogenous Fosl1 expressed under pathophysiological conditions, and from studies of Fosl1 deficiency in macrophages. Fosl1-overexpressing mice have a milder increase in serum inflammatory cytokine in response to lipopolysaccharide- and milder dextran sulfate sodium–induced colitis.[68] In macrophages and fibroblasts, enforced Fosl1 overexpression was associated with reduced nuclear factor κB activity and MAPK activation that would be consistent with the anti-inflammatory role observed in proximal tubular cells, but do not provide information on the role of physiological levels of endogenous Fosl1. Interestingly, colitis results in decreased kidney Klotho expression.[62] However, enforced overexpression of both Fosl1 and JunB in transgenic mice resulted in susceptibility to collagen-induced arthritis and an increase in T helper 17 cell numbers and inflammatory cytokine production.[29] In this regard, Fosl1-deficient macrophages had an enhanced Arg1 activity and *in vivo* Fosl1 deficiency from macrophages resulted in decreased arthritis.[30] Thus, the pro- or anti-inflammatory effects of Fosl1 described so far may depend on specific cell types, coexpressed Jun proteins, or abnormally high Fosl1 levels and have not explored cells with a high Klotho expression such as tubular cells (Supplementary Table S5).

Several limitations should be acknowledged. Given the already large spontaneous overexpression of Fosl1 in AKI, the impact of further upregulation of Fosl1 was not addressed. We characterized in detail a novel target for Fosl1, that is, Klotho. However, other genes may be targeted by this transcription factor, as suggested by prior enhancer analysis studies.[66] Additionally, further studies should be undertaken to confirm clinical translation relevance. While interventional studies cannot currently be performed, an analysis of the

impact of *FOSL1* gene variants on the risk of kidney disease and AKI should be explored in large datasets.

In conclusion, Fosl1 plays an adaptive, kidney protective role in proximal tubular cells in AKI and contributes to preserve the expression of the kidney protective, anti-inflammatory, antifibrotic, and antiaging molecule Klotho (Figure 9). Ongoing research on the role of Fosl1 in malignancy should consider this nephroprotective role of Fosl1 when developing novel therapeutic approaches. In this regard, the combination of cisplatin with approaches targeting Fosl1 need a careful follow-up regarding the nephrotoxicity potential.

DISCLOSURE

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AUTHOR CONTRIBUTIONS

MDS-N and AO conceived the original idea and supervised the research. MDS-N designed the research studies. LC, MR, and MIC performed the experiments and analyzed data. APC helped with the animal models. BSA contributed to the chromatin immunoprecipitation studies. SC helped with the human studies, and PCO performed the histological quantification. AD and EV performed the bioinformatics analysis. MDS-N and AO wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Figure S1. Fosl1 immunohistochemistry in cisplatin acute kidney injury (AKI).

Supplementary Figure S2. c-Jun is increased in murine cisplatin-induced and folic acid–induced acute kidney injury (AKI).

Supplementary Figure S3. Fosl1 expression and impact of proximal tubular cell Fosl1 deficiency in murine folic acid (FA)–acute kidney injury (AKI).

Supplementary Figure S4. Kidney FOSL1 in human samples.

Supplementary Figure S5. Kidney Fosl1 mRNA in healthy control wild-type (WT) and Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$)–deficient mice.

Supplementary Figure S6. Immunophenotyping of Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$)–deficient mice.

Supplementary Figure S7. Histological assessment and tumor necrosis factor-α (TNF-α) immunostaining in wild-type (WT) cisplatin–acute kidney injury (AKI) and Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$) cisplatin AKI mice.

Supplementary Figure S8. Kidney RNA-sequencing (RNA-Seq) transcriptomics comparing wild-type (WT) cisplatin–acute kidney injury (AKI) and Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$)–deficient cisplatin-AKI mice.

Supplementary Figure S9. A more severe decrease in kidney Klotho is an early event in of Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$)–deficient mice with cisplatin–acute kidney injury (AKI).

Supplementary Figure S10. Plasma levels of phosphate and phosphate regulators related to Klotho availability in Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$) and wild-type (WT) mice with cisplatin–acute kidney injury (AKI).

Supplementary Figure S11. Enrichment of Fosl1 binding sites in the murine and human Klotho promoters.

Supplementary Figure S12. The systemic administration of recombinant Klotho decreases the severity of cisplatin–acute kidney injury (AKI) in Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$) mice at 72 hours.

Supplementary Figure S13. U0126 dose-dependently inhibits extracellular signal–regulated kinases 1 and 2 (ERK1/2) phosphorylation and downstream effects of tumor necrosis factor superfamily, member 12 (TWEAK) on inflammatory mediators and Klotho in cultured tubular cells.

Supplementary Figure S14. Impact of Fosl1 overexpression on activator protein-1 (AP-1) activity in cultured proximal tubular cells.

Supplementary Figure S15. Impact of Fosl1 silencing on cytokine-induced regulated on activation, T-cell expressed, and secreted (RANTES) and tumor necrosis factor-α (TNF-α) expression in cultured proximal tubular cells.

Supplementary Table S1. Top-10 upregulated transcription factors according MSIG database in cisplatin kidney transcriptomics in wild-type mice with acute kidney injury (AKI) compared with wild-type controls with AKI at 24 hours.

Supplementary Table S2. Top-20 transcription factors in cisplatin–acute kidney injury (AKI) at 24 hours according to ChIP-X enrichment analysis 3 (ChEA3) transcription factor enrichment analysis.

Supplementary Table S3. Expression of genes encoding canonical components of the activator protein-1 (AP-1) transcription factor in cultured proximal tubular cells exposed to 100 ng/ml tumor necrosis factor superfamily, member 12 (TWEAK) or in kidneys from mice with acute kidney injury (AKI) induced by folic acid or cisplatin.

Supplementary Table S4. Kidney transcriptomics profiles of Fosl1 deficiency in proximal tubular cells (*Fosl1*$^{\Delta tub}$) and wild-type (WT) mice with cisplatin–acute kidney injury (AKI).

Supplementary Table S5. Fosl1 can behave as a repressor or promoter of gene transcription depending on cell context and the composition of activator protein-1 (AP-1) dimers.

Supplementary Methods.

Supplementary References.

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