

Specific deletion of glycogen synthase kinase-3 β in the renal proximal tubule protects against acute nephrotoxic injury in mice

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Renal proximal tubular damage and repair are hallmarks of acute kidney injury. As glycogen synthase kinase-3 β (GSK3 β) is an important cellular regulator of survival and proliferation, we determined its role during injury and recovery of proximal tubules in a mercuric chloride-induced nephrotoxic model of acute kidney injury. Renal proximal tubule-specific GSK3 β knockout mice exposed to mercuric chloride had improved survival and renal function compared to wild-type mice. Apoptosis, measured by TUNEL staining, Bax activation, and caspase 3 cleavage, was reduced in the knockout mice. The restoration of renal structure, function, and cell proliferation was also accelerated in the GSK3 β knockout mice. This enhanced repair, evidenced by increased Ki-67 and BRDU staining, along with increased cyclin D1 and c-myc levels, was recapitulated by treatment of wild-type mice with the small-molecule GSK3 inhibitor TDZD-8 following injury. This confirmed that hastened repair in the knockout mice was not merely due to lower initial injury levels. Thus, inhibition of GSK3 β prior to nephrotoxic insult protects from renal injury. Such treatment after acute kidney injury may accelerate repair and regeneration.

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Acute kidney injury (AKI) is characterized by an abrupt loss of renal function caused by ischemia–reperfusion or nephrotoxic insult and increases the risk of later chronic kidney disease.^{1–4} AKI involves a complex series of events that leads to tissue injury including endothelial and epithelial cell death, intra-tubular obstruction, changes in local microvascular blood flow, and inflammatory processes.⁵ The epithelial cells of proximal tubules are most susceptible to injury, as they have a high metabolic rate and greater ability to take up and concentrate toxins from both the luminal and the basolateral sides.^{6,7} However, these cells also have an amazing capacity to regenerate.^{1,8} Glycogen synthase kinase-3 (GSK3) is a serine/threonine protein kinase that is well positioned to coordinate multiple signaling pathways that regulate various cellular processes including gene transcription, translation, cytoskeletal organization, cell cycle progression, and survival.^{9,10} GSK3 exists in two isoforms encoded by distinct genes, α and β . As GSK3 α and GSK3 β isoforms share 98% sequence homology in their kinase domains,¹¹ no truly isoform-specific GSK3 β inhibitors have been developed yet.^{12,13}

GSK3 β is widely expressed in the kidneys,^{14–19} and recent studies have identified a possible role for GSK3 β in renal tubular injury. Gene silencing of GSK3 β in cultured proximal tubular cells reduced ATP depletion-induced apoptosis.²⁰ Further, inhibition using GSK3 isoform nonselective inhibitors reduced injury in endotoxemia and ischemia–reperfusion-induced AKI.^{20–22} As these studies used systemic inhibition of GSK3 to examine renal injury, the specific role of GSK3 β in survival of the proximal tubules *per se* has remained unclear. In addition, the role of GSK3 β in repair and regeneration of proximal tubules in AKI has not been explored. GSK3 isoforms have a pivotal role in cell cycle progression in embryonic stem cells and other cultured cell types.¹⁰ Although the relative importance of GSK3 α and GSK3 β isoforms in proliferation is not clear, targeted global knockout (KO) of GSK3 β in mice resulted in hyperproliferation of cardiomyocytes during embryonic development, whereas mice with global KO of GSK3 α appeared to be

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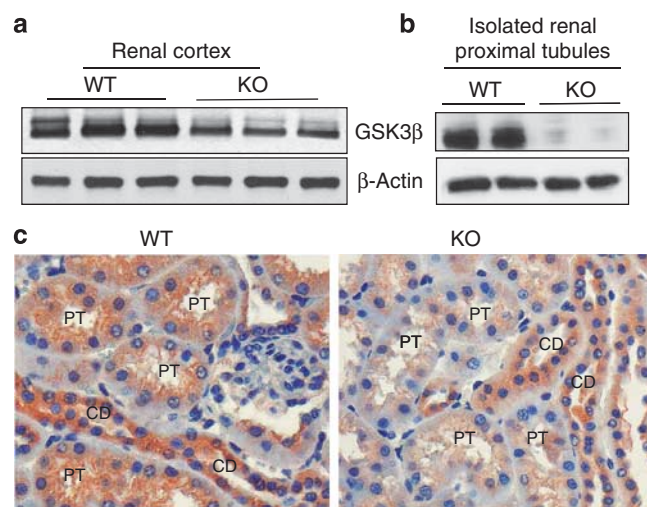


Figure 1 | Reduced expression of glycogen synthase kinase-3 β (GSK3 β) in renal proximal tubules of knockout (KO) mice. Western blot analysis shows reduced GSK3 β protein levels in tissue lysate of GSK3 $\beta^{\text{loxp/loxp}}$, γ -glutamyltranspeptidase (γ -GT)-Cre $^{+/+}$ (KO) compared with GSK3 $\beta^{\text{loxp/loxp}}$, γ -GT-Cre $^{-/-}$ (wild type (WT)) in (a) whole renal cortex and (b) acutely isolated proximal tubules from renal cortex. (c) Immunohistochemical staining shows reduced expression of GSK3 β in proximal tubules (PT) but not in the collecting ducts (CD) of KO mice (anti-GSK3 β antibody, $\times 200$ original magnification).

normal in this respect.²³ In a recent study, Peng *et al.*²⁴ reported that inhibition of GSK3 reduced migration of cultured proximal tubule cells in a scratch wound healing assay, suggesting suppression of wound healing in renal tubular cells. This result potentially contradicts the observations that inhibition of GSK3 is generally protective and reduces damage. Hence, in this study, we used proximal tubule-specific GSK3 β KO mice to examine the specific role of GSK3 β in tubular injury and repair in AKI.

We chose the widely used HgCl₂-induced model of AKI^{25–28} because mercury is a potent nephrotoxin, and its uptake via luminal γ -glutamyltranspeptidase (γ -GT) and the basolateral organic anion transporter system results in preferential accumulation and cytotoxicity of proximal tubules.^{25,29–34} Here, we report on the impact of selective genetic and chemical inhibition of GSK3 on initial damage and subsequent repair of renal proximal tubules in AKI.

RESULTS

Generating renal proximal tubule-specific GSK3 β KO mice

To obtain renal proximal tubule-specific gene deletion of GSK3 β , we bred GSK3 $\beta^{\text{loxp/loxp}}$ mice³⁵ with γ -GT-Cre $^{+/+}$ mice.³⁶ Mice progeny exhibited the expected Mendelian ratio, and histopathological examination revealed no renal abnormalities in the KO mice (GSK3 $\beta^{\text{loxp/loxp}}$, γ -GT-Cre $^{+/+}$) when compared with wild type (WT) (GSK3 $\beta^{\text{loxp/loxp}}$, γ -GT-Cre $^{-/-}$). Western blot analysis of lysate from whole renal cortex (Figure 1a) and isolated proximal tubules (Figure 1b) showed significantly reduced levels of GSK3 β expression in the KO mice compared with WT mice. Immunohistochemical staining with antibodies selective for GSK3 β showed

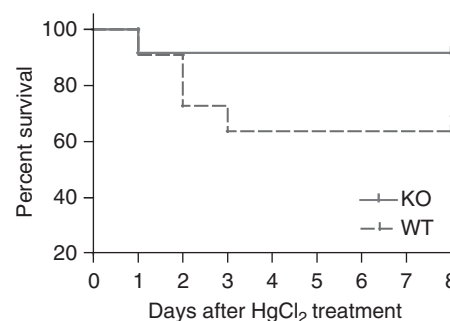


Figure 2 | Knockout (KO) mice show higher survival rate following HgCl₂ treatment. Kaplan-Meier survival curve of wild-type (WT) and KO mice injected with a single subcutaneous injection of 8.14 mg/kg body weight of HgCl₂. * $P < 0.01$, $n = 20$ per group.

significantly reduced levels of GSK3 β in the proximal tubules but not in other nephron segments of KO mice (Figure 1c).

Proximal tubule-specific KO of GSK3 β reduces HgCl₂-induced mortality and tubular injury

When treated with 8.14 mg/kg body weight of HgCl₂, a 10% mortality rate was observed in both WT and KO groups within 24 h (Figure 2). By days 2–3, the mortality rate further increased to 40% in the WT group, whereas it remained unchanged in the KO cohort, indicating that GSK3 β gene deletion in the proximal tubule reduced HgCl₂-induced mortality (Figure 2). Kidneys showed tubular dilatation, cellular necrosis, and loss of brush border in both WT and KO mice by day 2 after HgCl₂ treatment (Figure 3a). Semiquantitative injury score showed that on day 2 the injury score in the KO mice was only half of that in WT mice (Figure 3b). The injury score did not change significantly in WT mice by day 4 and remained 16-fold higher than baseline values on day 6. In the KO mice, the injury score decreased by 1.8-fold on day 4 and attained baseline levels by day 6. Blood urea nitrogen (BUN) levels increased by 7.8-fold in WT and 3.5-fold in the KO group by day 2 after HgCl₂ treatment compared with baseline (20 ± 6 vs. 155 ± 15 mg/dl in WT and 25 ± 7 vs. 87 ± 18 mg/dl in KO). By days 4 and 6, BUN levels in the KO mice were reduced by 42% and 60%, respectively, returning to levels close to the baseline. In contrast, BUN levels remained essentially unchanged in the WT group on day 4, followed by a 40% decrease on day 6, but failed to reach baseline levels even by day 8 (Figure 3c). Plasma creatinine levels showed a similar pattern (Figure 3d).

Reduced apoptosis in the renal cortex of GSK3 β KO mice following HgCl₂ treatment

To examine whether the reduced levels of injury in the KO mice could be attributed to a decreased rate of apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out. Within 24 h after HgCl₂ treatment, 12% of nuclei were TUNEL positive in the WT kidney compared with only 4% in KO (Figure 4a and b). These apoptotic cells were mostly in

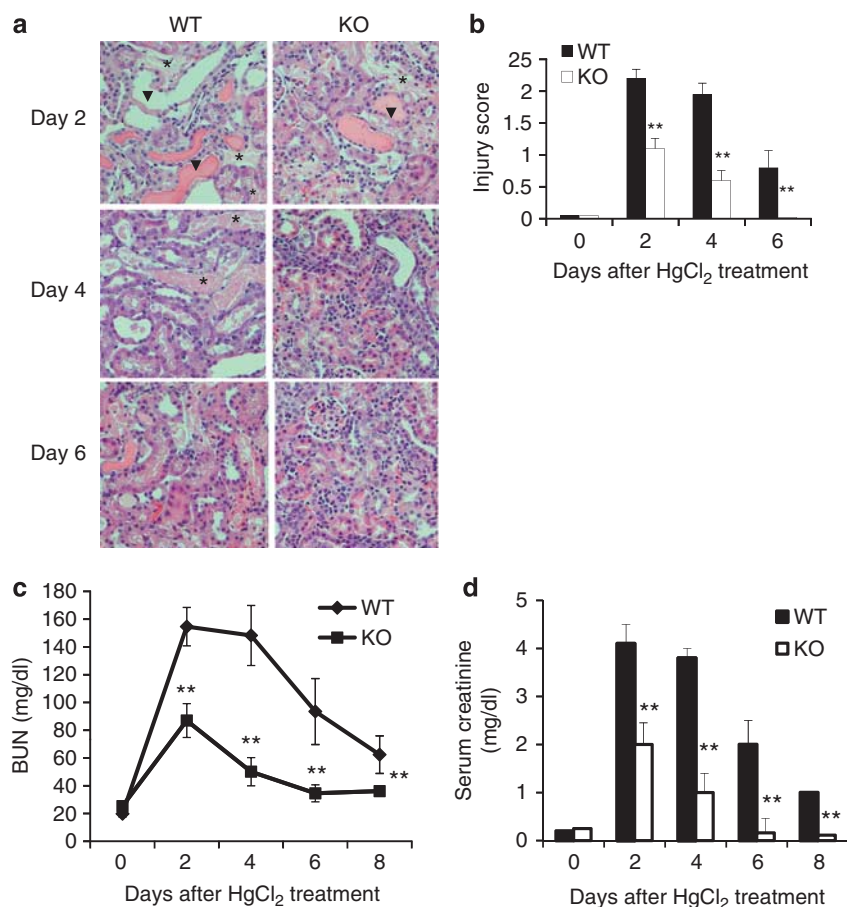


Figure 3 | Proximal tubule-specific glycogen synthase kinase-3 β (GSK3 β) gene deletion reduced HgCl₂-induced renal injury.

(a) Tubular injury is manifest as necrotic tubules (*), flattened epithelium (arrows), and loss of nuclei (arrows), and was less severe in knockout (KO) compared with wild type (WT) at all-time points (hematoxylin and eosin, $\times 200$ original magnification). (b) Injury score of renal tissue sections. $**P < 0.001$, $n = 5$ per group. (c) Blood urea nitrogen (BUN) and (d) serum creatinine levels are lower in KO mice. $**P < 0.001$, $n = 21$ per group.

proximal tubules as shown by an immunoperoxidase-labeled TUNEL assay (Supplementary Figure S1 online). This result indicated that apoptosis was associated with tissue injury early in HgCl₂-induced AKI, and the rate of apoptosis was reduced in KO mice.

To determine the mechanism underlying the antiapoptotic effect of GSK3 β gene deletion, we examined protein levels of pro-apoptotic- activated Bax, cleaved caspase 3, and an antiapoptotic factor Bcl 2. Although a time-dependent increase in activated Bax levels was observed in both WT and KO mice, the levels were significantly lower in KO mice (Figure 4c). Activated Bax levels in the KO mice were lower by 2- and 2.8-fold, respectively, on days 1 and 2 when compared with WT mice (Figure 4d). Total Bax levels showed no change. Cleaved caspase 3 levels peaked on day 1, increasing by fivefold compared with day 0 levels. Compared with WT mice, in KO mice cleaved caspase 3 levels were five- and twofold lower on days 1 and 2, respectively (Figure 4c and e). In the WT group, Bcl 2 levels were reduced by 20% and 40%, respectively, by days 1 and 2, compared with day 0. In the KO mice, no significant difference was

observed (Figure 4c and f). These data demonstrate that the KO mice were protected from the apoptosis caused by HgCl₂.

Accelerated cell proliferation in renal proximal tubules of KO mice

We next determined whether GSK3 β gene deletion in proximal tubule cells alters their proliferative response after injury. Ki-67 nuclear staining, a proliferative marker, was higher by 2-fold on day 2 and 3.5-fold on day 4 in the KO group compared with the WT group (500-fold more than baseline) (Figure 5a and b). 5-bromo-2'-deoxyuridine (BRDU)-positive nuclei were also 2.5-fold higher in the KO kidney on day 4 compared with WT (Figure 5c and d). Both Ki-67 and BRDU staining decreased in KO kidneys after day 4, consistent with the improved renal function and decreased histopathology injury data. Triple immunofluorescence staining of kidneys on day 4 showed that the dividing cells, indicated by proliferating cell nuclear antigen staining, were more abundant in the KO mice, and were primarily located in the proximal tubule (stained with Lotus tetragonolobus agglutinin) (Figure 5e).

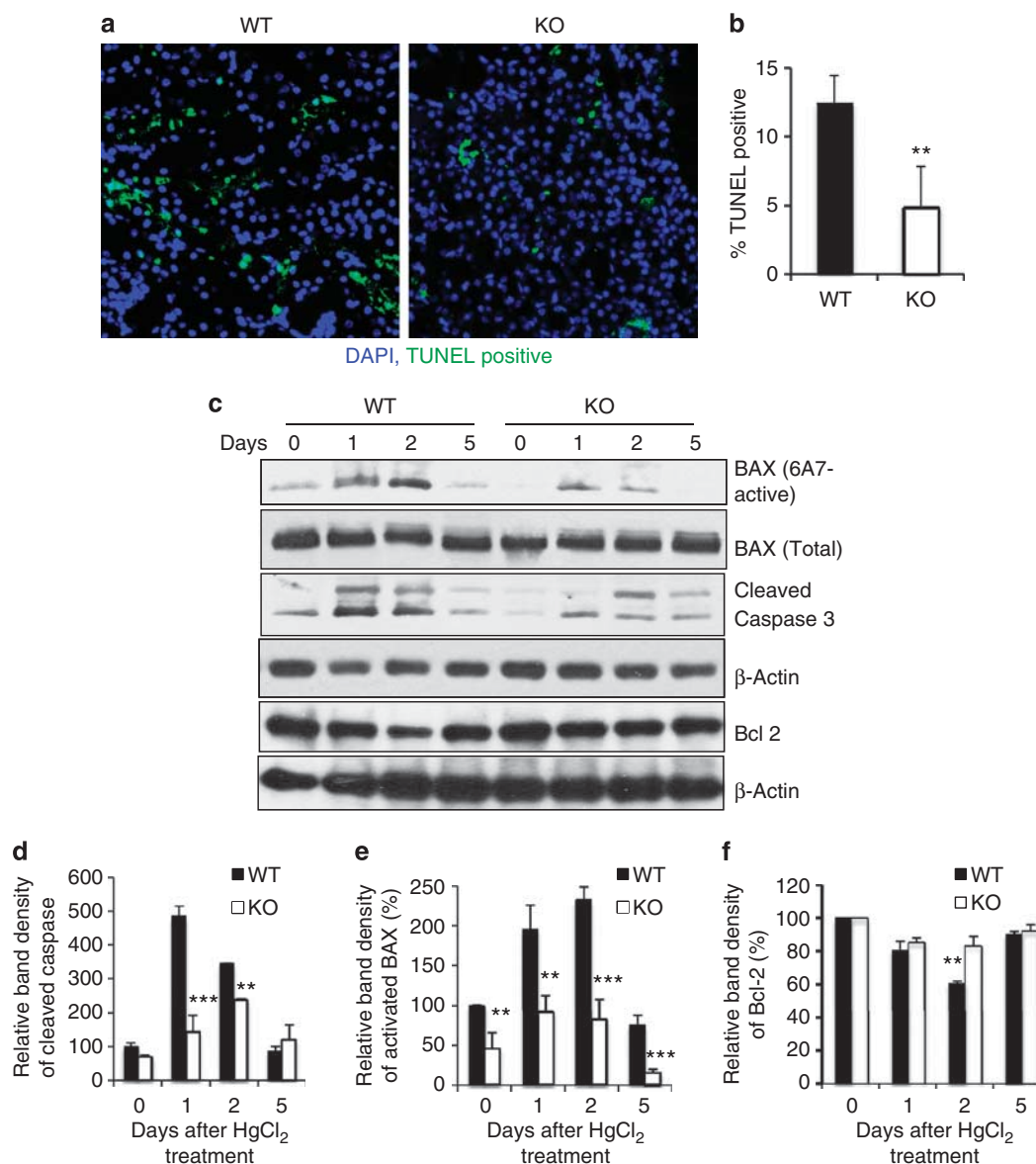


Figure 4 | HgCl₂-induced apoptosis is reduced in knockout (KO) mice. (a) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) nuclear staining in renal cortex 24 h after HgCl₂ treatment and (b) quantification, which shows reduced TUNEL-positive nuclei in the KO group ($n = 5$ per group). (c) Cleaved caspase 3 and activated Bax levels are lower by western blot in KO kidney compared with wild type (WT) (d, e, f). ** $P < 0.001$, *** $P < 0.0001$. (d, e, f) Based on two time-course studies. DAPI, 4,6-diamidino-2-phenylindole.

To examine the mechanism for the higher rate of proliferation in the KO mice, protein levels of the proliferative factors, cyclin D1, c-myc, and β -catenin, known to be regulated by GSK3 β were measured. Cyclin D1 and c-myc levels in the kidney cortex were markedly upregulated in KO mice compared with WT mice, especially on days 1–4 (Figure 6a). Interestingly, cyclin D1 levels decreased by day 6 in the KO mice, whereas in the WT mice the highest cyclin D1 levels were observed on days 6 and 8 (Figure 6b). c-myc levels were significantly higher in KO mice on days 3, 4, and 6 compared with that in WT mice (Figure 6c). β -Catenin levels did not differ significantly between the WT and KO groups. These results indicated an accelerated rate of proliferation in KO mice when compared with WT mice. Examination of renal

fibrosis by Sirius red staining, a marker of collagen, revealed no significant difference between the WT and KO mice 8 days after HgCl₂ treatment, although it is possible that fibrosis occurs after a longer period of time (Supplementary data).

Paired study shows that GSK3 β inhibition accelerates proliferation

To probe further the role of GSK3 β in regeneration following AKI, we tested the effect of TDZD-8, a small-molecule inhibitor of GSK3 β .^{37,38} In this study, TDZD-8 treatment was carried out after onset of injury in WT mice. WT mice were first administered 6.3 mg/kg of HgCl₂. On day 2 (48 h after HgCl₂), BUN levels were measured and mice with approximately 82 ± 2 mg/dl were separated into two groups. One

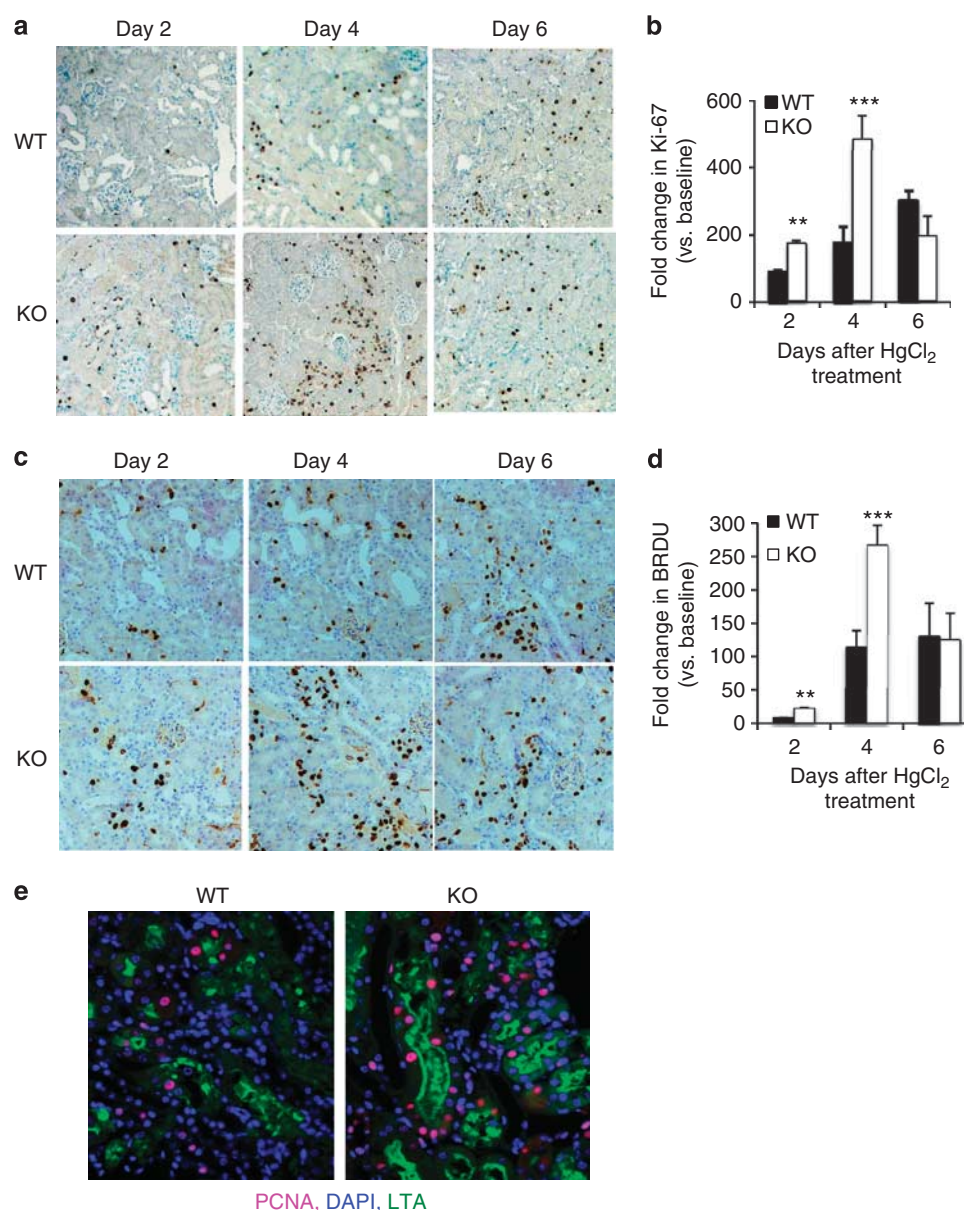


Figure 5 | Proliferation is accelerated in knockout (KO) kidneys. Immunohistochemical staining and quantification for (a, b) Ki-67 and (c, d) BRDU show more proliferating cells in KO kidneys. $^{**}P < 0.001$. $^{***}P < 0.0001$, $n = 5$ per group, $\times 200$ original magnification. Immunofluorescence staining for proliferating cell nuclear antigen (PCNA) and Lotus tetragonolobus agglutinin (LTA) shows more dividing cells in proximal tubule cells of KO mice on day 4 after HgCl₂ treatment (e); $\times 400$ original magnification. DAPI, 4,6-diamidino-2-phenylindole, WT, wild type.

group was administered vehicle and the other TDZD-8 (1 and 0.5 mg/kg, respectively, on days 2 and 3 after HgCl₂ treatment). In the TDZD-8-treated group, BUN levels decreased significantly compared with the vehicle-treated group (vehicle, 72 ± 9 vs. TDZD-8, 51 ± 6 mg/dl on day 4, $n = 9$ and vehicle, 51 ± 8 vs. TDZD-8, 30 ± 7 mg/dl on day 6, $n = 6$) (Figure 7a). TDZD-8-treated mice also showed 40% and 36% lower kidney injury scores compared with vehicle-treated mice on days 4 and 6, respectively (Figure 7b and c), indicating that inhibition of GSK3 accelerated regeneration. Further, protein levels of cyclin D1, c-myc, and β -catenin were significantly higher on days 3 and 4 in the TDZD-8-treated compared with the vehicle-treated mice (Figure 8a).

By day 4, the number of BRDU-positive nuclei in TDZD-8-treated mice was almost double when compared with vehicle-treated mice (Figure 8b and c). Similarly, Ki-67 staining was more than double in the TDZD-8-treated mice, compared with the vehicle-treated mice on day 4 (Figure 8d and e). Sirius red staining, on tissue sections, 8 days after HgCl₂ treatment revealed no significant change in fibrosis between the two groups at this time point (Supplementary data).

Regulation of renal GSK3 β activity in response to HgCl₂ treatment

We next examined whether GSK3 β activity was regulated in HgCl₂-induced AKI. Protein levels of pGSK3 β , the inhibited

form of GSK3 β phosphorylated at serine 9, decreased to undetectable levels 6 h after HgCl₂ injection, gradually increasing by 16 h (Figure 9a). By 32 h, pGSK3 β levels increased above baseline levels and remained high for 5 days. Protein levels of β -catenin, a negatively regulated substrate of GSK3, also increased by 48 h, consistent with the GSK3 inhibition. These data indicated that, following HgCl₂ treatment, GSK3 β (and likely also GSK3 α) was initially activated and then subsequently inhibited.

DISCUSSION

Our studies demonstrate that GSK3 β activity in the renal proximal tubules modulates injury and repair in HgCl₂-induced AKI. Proximal tubule-specific KO of GSK3 β improved survival

in HgCl₂-treated mice by reducing nephrotoxicity and injury, as well as accelerating cell proliferation and repair. These studies show the broader benefits of GSK3 β gene deletion or inhibition on reducing injury and, more importantly, on repopulating renal proximal tubules after AKI.

Renal tubular injury is a distinctive feature of AKI, characterized by apoptosis and necrosis of renal tubules. The extent of injury in our study was similar to previously reported *in vivo* patterns of HgCl₂-induced nephrotoxicity,^{27,28} insofar as the onset of toxic effects was very rapid and injury was accompanied by apoptosis. Compared with WT mice, the KO mice had better renal function following HgCl₂ treatment with lower BUN and plasma creatinine levels. This was linked to less severe injury and accelerated repair observed in KO mice. The reduced injury itself could be at least partially due to a reduced rate of apoptosis in the KO mice.

GSK3 β is known to regulate the intrinsic mitochondrial apoptotic pathway.³⁹ Bax, a pro-apoptotic protein that is constitutively expressed in the cell, is activated by phosphorylation by GSK3 β .⁴⁰ The activated Bax oligomerizes with the mitochondrial outer membranes and forms pores leading to the release of cytochrome *c* and other pro-apoptotic factors, which in turn activate caspase-dependent or -independent pathways.^{5,41–43} In our study, activated Bax levels and cleaved caspase 3 levels were lower in the KO mice compared with WT mice. Similarly, Bcl 2, an antiapoptotic protein, remained unchanged in KO mice, whereas it was reduced in the WT mice following HgCl₂ treatment. Bcl 2 is cleaved/degraded by caspase 3 during apoptosis, which in turn leads to mitochondrial pore formation.⁴⁴ Earlier studies demonstrated that systemic GSK3 inhibition could reduce apoptosis associated with endotoxemia^{21,22} and ischemia-reperfusion models of AKI.²⁰ Although consistent with these studies, our results clearly demonstrated that gene deletion of GSK3 β in the proximal tubule *per se* reduces apoptosis and renal injury and improves survival after AKI.

GSK3 β is constitutively active in resting cells and undergoes a rapid and transient inhibition in response to a number of external signals. GSK3 β activity increased within 6 h of HgCl₂ treatment, which coincided with the increased levels of

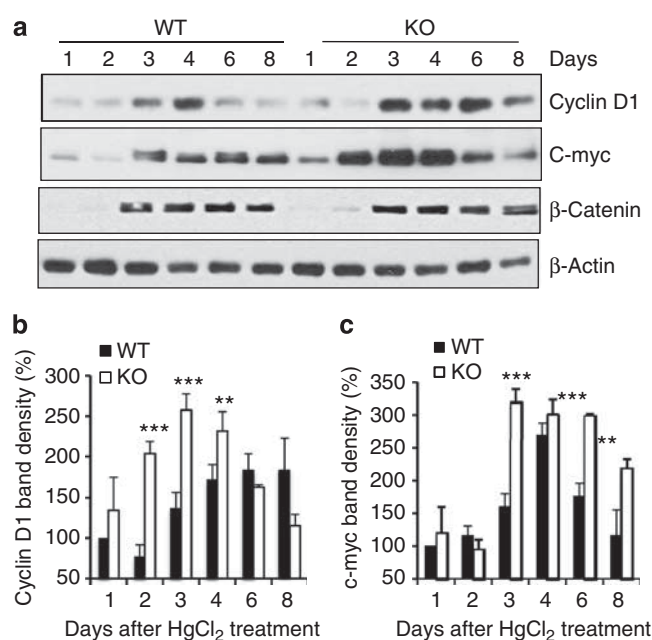


Figure 6 | Cyclin D1 and c-myc levels are higher in knockout (KO) kidneys. (a) Western blot analysis and (b, c) quantification of band density for protein levels of cyclin D1, c-myc, and β -catenin. (b, c) Based on two time-course studies. ** $P < 0.001$, *** $P < 0.0001$. Data are expressed as mean \pm s.d. WT, wild type.

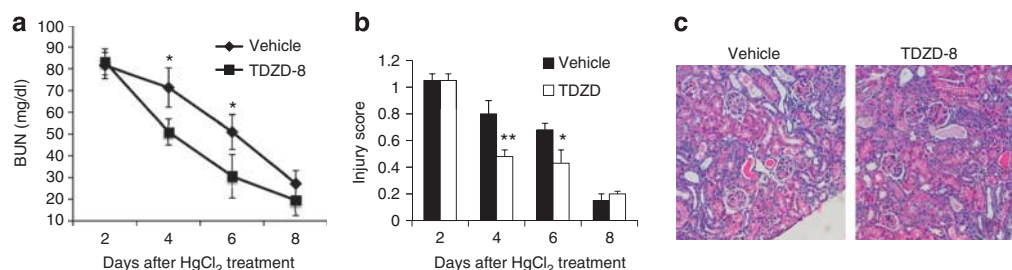


Figure 7 | Recovery is faster in glycogen synthase kinase-3 (GSK3) inhibitor-treated mice compared with similarly injured, but vehicle-treated, mice. Wild-type (WT) mice were injected once with 6.3 mg/kg HgCl₂. After 48 h, mice with comparable levels of blood urea nitrogen (BUN) were administered vehicle or GSK3 inhibitor, TDZD-8 (1 mg/kg body weight (BWt) on day 2 and 0.5 mg/kg BWt on day 3). (a) BUN levels decreased at a faster rate in TDZD-8-treated mice. (b) Injury score shows reduced injury in TDZD-8-treated mice, * $P < 0.01$. ** $P < 0.001$, $n = 12$ per group. (c) Representative image of renal cortex of WT and TDZD-8-treated mice 4 days after HgCl₂ treatment (hematoxylin and eosin, $\times 100$ original magnification).

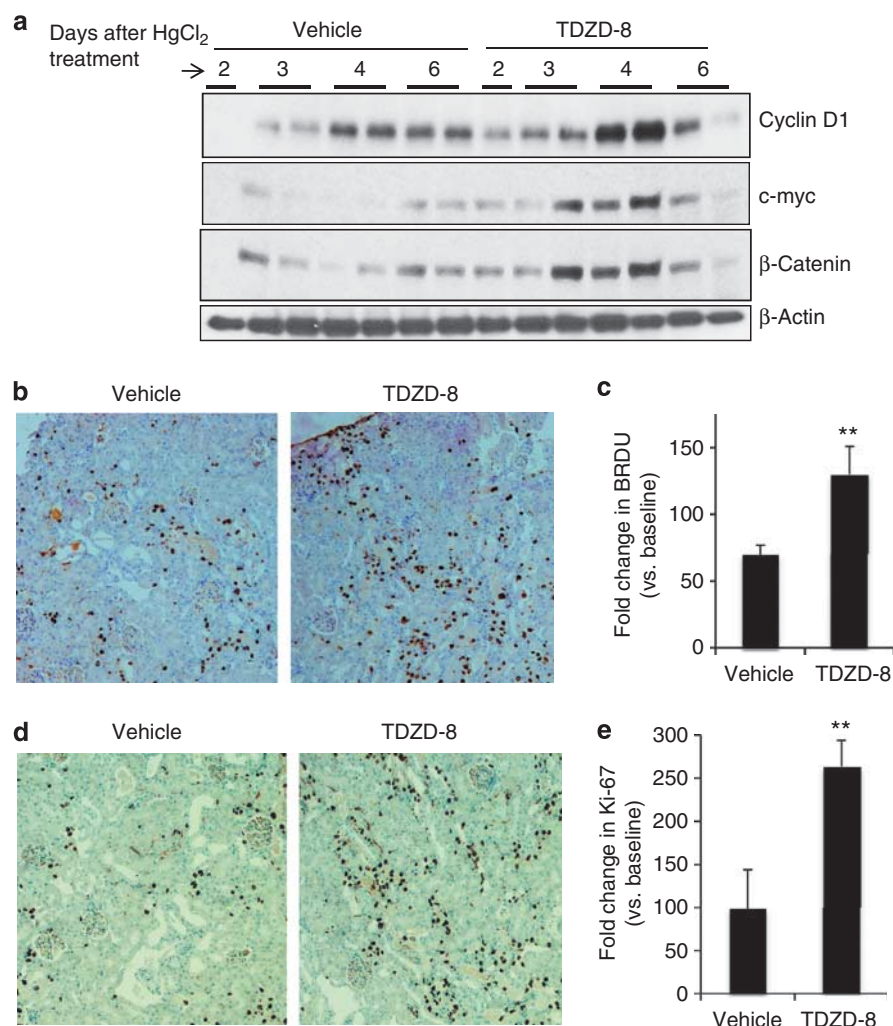


Figure 8 | Proliferation is accelerated in glycogen synthase kinase-3 β (GSK3 β) inhibitor-treated mice compared with similarly injured, but vehicle-treated, mice. (a) Cyclin D1, c-myc, and β -catenin levels by western blot from renal cortical protein lysates were higher in TDZD-8-treated mice. **(b, c)** BRDU immunostaining and quantification of positive nuclei and **(d, e)** Ki-67 immunostaining and quantification of positive nuclei are increased in TDZD-8-treated mice on day 4 after HgCl₂ treatment. ****** $P < 0.001$, $\times 200$ original magnification.

apoptosis and injury seen in WT mice (Figure 8). These results are consistent with similar findings in a rat model of ischemia–reperfusion-induced AKI.²⁰ Surprisingly, in our study, GSK3 β activity was reduced to below pre-HgCl₂ treatment levels by 32 h and stayed low for 5 days. This period of time (2–5 days after HgCl₂ treatment) coincides with a period of active cell proliferation and recovery in WT mice, suggesting that suppression of renal GSK3 β activity could be a natural mechanism employed by renal tubules undergoing regeneration following injury. Hence, it could be possible that in the WT mouse kidney GSK3 is normally inhibited to some extent by the phosphatidylinositol 3 kinase-AKT, protein kinase A, epidermal growth factor receptor, and Wnt signaling pathways,^{45–48} which are known to be important for cell proliferation and tissue repair.^{28,49–52} This response, however, might not be adequate to prevent severe AKI and mortality. Our findings demonstrate that proximal tubule-specific gene KO of GSK3 β or the use of a

GSK3 inhibitor further significantly protected against injury and accelerated the repair process.

A rapid proliferative response leading to the restoration of nephron structure and function is very important, because episodes of AKI are not always fully reversible and may lead to chronic kidney disease. An interesting observation was that repair occurred at a linear rate in the WT kidney, but at an exponential rate in the KO kidney. On the basis of injury scores, BUN/creatinine levels and cell proliferation, the phase of renal repair began on day 2 and peaked on day 4 in KO mice, whereas it started later in WT mice at days 4–6 after initial injury. A paired study using similarly injured WT mice showed that TDZD-8 treatment improved recovery of renal structure and function and the rate of proliferation compared with vehicle-treated mice, thus demonstrating that the accelerated rate of repair in KO mice could not be attributed to reduced levels of initial renal injury alone. Cyclin D1, c-myc, and β -catenin are pro-proliferative factors and also

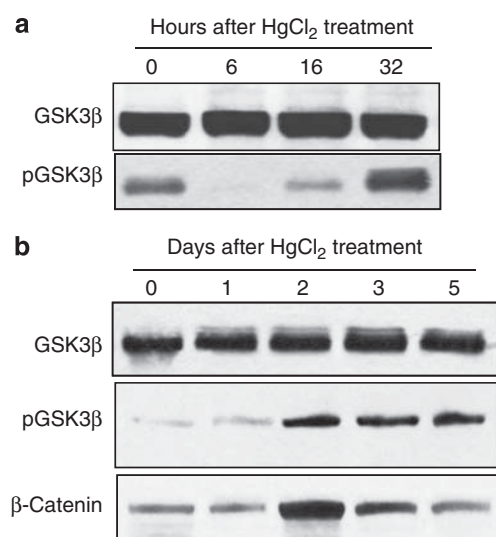


Figure 9 | Renal glycogen synthase kinase-3 β (GSK3 β) activity changes in response to HgCl₂ treatment. Western blot analysis of tissue lysates from whole renal cortex of wild-type (WT) mice injected once with 8.14 mg/kg of HgCl₂. (a) Inactive phosphorylated GSK3 β levels initially decreased within 6 h after HgCl₂ treatment and (b) increased to above baseline by 24 h. β -Catenin levels also increased on days 2–5.

substrates of GSK3 β . Phosphorylation of cyclin D1, c-myc, and β -catenin by GSK3 β destabilizes them, leading to ubiquitination and degradation.^{48,53,54} We found an accelerated rate of increase in cyclin D1 and c-myc accumulation in the GSK3 β KO mice compared with the WT mice. This was confirmed in studies using TDZD-8. Although β -catenin levels were significantly higher in the TDZD-8-treated mice than in the vehicle-treated mice, the KO mice did not show such a change. Past studies have suggested that gene deletion of at least three of the four alleles of both isoforms of GSK3 α versus β are required to show an appreciable change in β -catenin levels.⁵⁵ It could also be possible that administration of HgCl₂ by itself can increase β -catenin accumulation by activating Wnt signaling.

Our observations on the rapid regenerative response in GSK3 β -inactivated renal proximal tubules could be important in light of the current theory that it is the surviving renal tubular epithelial cells that proliferate and repopulate tubules in AKI.⁸ In WT embryonic stem cells, GSK3 inhibition has been suggested to help in maintaining self-renewal and pluripotency,^{56,57} and the inhibition of GSK3 has been reported to enhance repopulation of the bone marrow by hematopoietic stem cells.⁵⁸ Hence, inhibition of GSK3 β could be a key switch that turns on cell proliferation and regeneration of renal proximal tubules in AKI, thus warranting further studies.

In summary, GSK3 β mediates injury and repair in HgCl₂-induced AKI. Although kidneys appear to have an innate ability to suppress GSK3 β activity in order to reduce injury and promote regeneration of tubular cells, it is not sufficient to protect mice from a high rate of mortality under conditions of severe injury. Proximal tubule-specific KO of GSK3 β not only reduced tubular injury but also accelerated tubular

regeneration. Our study also showed that the mechanism of GSK3 β protection is specific to the kidney, as opposed to being a systemic effect that could have indirectly ameliorated organ damage. Thus, GSK3 β could be a major regulator of recovery from acute toxic kidney injury. This is clinically relevant as it provides a possible treatment option to accelerate recovery of established AKI. Thus, these findings have potential therapeutic application for a broad range of kidney diseases.

MATERIALS AND METHODS

Generating KO mice

GSK3 $\beta^{\text{loxP/loxP}}$ mice were bred with transgenic mice in which Cre expression was driven by a promoter fragment of γ -GT, expressed in the proximal tubule.³⁶ Mice were on a pure C57/BL6J background. Genotype was determined by PCR using tail DNA and primers described earlier.^{17,36} Proximal tubules were isolated from WT and KO mice renal cortex by collagenase digestion, followed by percoll gradient high-speed centrifugation.^{59,60}

AKI studies

Female WT and KO mouse littermates, 10 weeks old and weighing 24–25 g, were injected once subcutaneously with HgCl₂ at a nephrotoxic dose of 8.16 mg/kg body weight.²⁷ Mice were killed to examine renal morphology and protein expression, and blood plasma was collected for BUN and creatinine measurements. For measurement of BRDU incorporation, mice received an injection 2 h before killing. In a substudy (Figure 7), only WT mice were injected with 6.3 mg/kg of body weight of HgCl₂. Plasma BUN levels were measured on day 2 and mice with comparable levels of BUN were separated into two groups ($n = 12$ in each group). One group received two intraperitoneal injections of an ATP noncompetitive GSK3 inhibitor, TDZD-8 dissolved in 75% dimethylsulphoxide (1 mg/kg on day 2 and 500 μ g/kg on day 3), and the other group received vehicle. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD) and were approved by the IACUC committee of Vanderbilt University.

BUN and creatinine measurements

BUN levels were measured using a QuantiChrom Urea Assay Kit from BioAssay Systems, Hayward, CA. Creatinine levels were measured using high-performance liquid chromatography at the Vanderbilt Hormone Assay Core.

Western blot analysis

Protein (20 μ g) was loaded on 12 or 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis mini-gels and immunoblotting was carried out as described earlier.¹⁷ Monoclonal antibody for GSK3 β (BD-Transduction Laboratories, San Jose, CA) and polyclonal antibodies for pGSK3 β , β -actin, cleaved caspase 3, Bax (Cell Signaling, Danvers, MA), and c-myc (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Immunohistochemistry and immunofluorescence

Kidneys were collected at the time of killing and a cross-section was immediately fixed in 4% paraformaldehyde at 4°C overnight. Samples were dehydrated in a graded alcohol series and embedded in paraffin for histological analysis. To unmask antigens, 5- μ m sections were boiled in Target Retrieval solution (Dako, Carpinteria, CA).

Polyclonal antibodies for GSK3 β (Santa Cruz Biotechnology), Ki-67, and BRDU (Cell Signaling) were used. IITL (Vector Laboratories, Burlingame, CA), proliferating cell nuclear antigen (Dako), and secondary antibody (Jackson ImmunoResearch Laboratories) conjugated to Cy3 were used. Ki-67-, BRDU-, and proliferating cell nuclear antigen-positive nuclei and total nuclei were counted manually at $20\times$, in 20 high-power fields for each kidney sample, and an average percent positivity per high-power field was calculated per kidney. Negative controls without primary antibody showed no staining. Images were captured with a Nikon Eclipse E600 microscope and a Nikon DXM1200 digital camera (Melville, NY). All assessments were blinded to the treatment protocols.

TUNEL assay

TUNEL assay was performed to evaluate apoptosis using an *In situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). In brief, 4- μ m renal sections were exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides, including tetramethylrhodamine-labeled dUTP. Total and TUNEL-positive nuclei were counted as described for proliferating cell nuclear antigen.

Tubule injury and fibrosis measurements

Tubular injury score was determined by assessing hematoxylin and eosin-stained paraffin sections. Loss of brush border, tubular dilation, cast formation, cell lysis, vacuolization, and sloughing were scored on a scale of 0–4, where 0 = no injury; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; and 4 = >75% tubules having necrosis, dilatation, or cell swelling. The observer was unaware of the protocol assignments of the mice. Fibrosis was measured by morphometric analysis of collagen fibril by Sirius red staining. Deparaffinized sections were stained with 0.1% picosirius red.⁶¹

Statistical analysis

Comparisons between WT and KO mice or TDZD-8-treated and vehicle-treated mice were analyzed by the unpaired Student's *t*-test. Comparisons of multiple points were made using analysis of variance with the Bonferroni correction. $P < 0.05$ was taken as significant. Data are expressed as means \pm s.e.m.

DISCLOSURE

All the authors declared no competing interests.

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Author contributions

This work was partly done at Vanderbilt University by RR and CH.

SUPPLEMENTARY MATERIAL

Figure S1. TUNEL staining of mice kidney 24 h after HgCl₂ treatment. Arrows indicate brown staining of TUNEL positive nuclei.

Figure S2. Sirius red staining of WT and KO mice kidney 8 days after HgCl₂ treatment. $\times 200$ original magnification.

Figure S3. Sirius red staining of kidneys of WT mice treated with vehicle or TDZD-8, 8 days after HgCl₂ treatment. $\times 200$ original magnification.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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