Acute kidney injury is a devastating disease with high morbidity in hospitalized patients and contributes to the pathogenesis of chronic kidney disease. An underlying mechanism of acute kidney injury involves ischemia-reperfusion injury which, in turn, induces oxidative stress and provokes organ damage. Nrf2 is a master transcription factor that regulates the cellular response to oxidative stress. Here, we examined the role of Nrf2 in the progression of ischemia-reperfusion injury-induced kidney damage in mice using genetic and pharmacological approaches. Both global and tubular-specific Nrf2 activation enhanced gene expression of antioxidant and NADPH synthesis enzymes, including glucose-6-phosphate dehydrogenase, and ameliorated both the initiation of injury in the outer medulla and the progression of tubular damage in the cortex. Myeloid-specific Nrf2 activation was ineffective. Short-term administration of the Nrf2 inducer CDDO during the initial phase of injury ameliorated the late phase of tubular damage. This inducer effectively protected the human proximal tubular cell line HK-2 from oxidative stress-mediated cell death while glucose-6-phosphate dehydrogenase knockdown increased intracellular reactive oxygen species. These findings demonstrate that tubular hyperactivation of Nrf2 in the initial phase of injury prevents the progression of reactive oxygen species-mediated tubular damage by inducing antioxidant enzymes and NADPH synthesis. Thus, Nrf2 may be a promising therapeutic target for preventing acute kidney injury to chronic kidney disease transition.

KEYWORDS: acute kidney injury; chronic kidney disease; ischemia-reperfusion; oxidative stress; transcription regulation

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Acute kidney injury (AKI) is a complex disorder that is defined as abrupt renal dysfunction, which is associated with high morbidity and mortality and is a major risk factor for chronic kidney disease (CKD). Therefore, the development of strategies for alleviating the severity and progression of AKI is urgently needed. Renal ischemia-reperfusion injury (IRI) is a major cause of AKI often brought on by critical illness or surgery. IRI produces various reactive oxygen species (ROS) that provoke tubular atrophy, endothelial dysfunction, and cell death through maladaptation of tubular cell repair. Because repeated tubular injury and maladaptive repair are considered major mechanisms linking AKI to CKD, it is proposed that activation of cellular defense systems against oxidative stress in kidneys is a promising strategy to ameliorate AKI.

Nrf2 (nuclear factor erythroid 2-related factor 2) is a master transcriptional regulator of cellular defenses against oxidative stress. Under normal conditions, Nrf2 is continuously sequestered by its negative regulator Keap1 (Kelch-like ECH-associated protein 1) and is degraded through the ubiquitin-proteasome pathway. Under oxidative stress, Keap1 is inactivated, and Nrf2 escapes from degradation. Stabilized Nrf2 proteins form heterodimers with small Maf protein in nuclei and induce target gene expression for antioxidant/electrophile response elements. Nrf2 has been proposed as a therapeutic target for AKI, and the evidence that pharmacological activation of Nrf2 ameliorates AKI severity is largely undefined. However, the long-term consequences of Nrf2 activation on AKI to CKD transition are largely undefined.
approaches in mice with IRI. Our results demonstrate that Nrf2 hyperactivation enhances a cell-autonomous defense system against oxidative stress in tubular cells during the early stage of injury and effectively prevents both tubular damage during the initial injury stage and damage expansion during the late injury stage. Synchronized induction of antioxidant enzymes and reduced nicotinamide adenine dinucleotide phosphate (NADPH) synthesis enzymes in tubules by Nrf2 is associated with these protective effects. Notably, early short-term treatment with a chemical inducer of Nrf2 after initial injury appears to be crucial for this protection, demonstrating that tubular Nrf2 activation is a promising strategy to prevent the AKI-CKD transition.

RESULTS
Nrf2 ameliorates IRI-induced tubular damage progression
To evaluate the long-term consequences of initial injury,7,18 histological analyses of kidneys from wild-type (WT) mice at 14 days after unilateral IRI treatment (day 14) were conducted. Elastica-Masson staining showed that all tubules in contralateral noninjured kidneys were strongly stained red, while weakly stained (pale/white) areas spread over IRI kidneys (Figure 1a). The analyses of hematoxylin-eosin staining and F4/80 immunostaining of serial sections showed that the areas with weak Elastica-Masson staining were injured tubular areas with tubular atrophy and immune cell infiltration (Figure 1b and c), which could be distinguished from well-preserved tubular areas stained red as reported previously.19 Time-course analyses of IRI kidneys showed that the injured tubular area gradually expanded until day 7, and then the expanded area remained unchanged until at least day 14 (Supplementary Figure S1A–C). The electron microscopy analysis demonstrated that the number of mitochondria dramatically decreased in atrophic tubular cells of IRI kidneys at day 14 (Supplementary Figure S1D). These data indicate that IRI-induced tubular damage shifts from the early progressive phase to the late conservative phase of injury around day 14 as previously observed.7

To analyze the roles of Nrf2 in the kidney damage progression, we analyzed Nrf2 knockout (Nrf2-KO) mice and hypomorphic Keap1 knockout (Keap1-KD) mice, in which Nrf2 is constitutively activated in the whole body.20,21 For the contralateral kidneys of these mutant mice at day 14, no abnormality was observed following Elastica-Masson staining (Figure 1a and d). At day 14, severe tubular damage was observed in IRI kidneys of Nrf2-KO mice compared to those of WT mice, and the damage was ameliorated in Keap1-KD mice (Figure 1a and d). Then, the function of the injured kidney from Keap1-KD mice was assessed by nephrectomy of the contralateral kidneys at day 14, leaving the mice with only IRI kidneys (Figure 1e). Strikingly, the plasma creatinine concentration was remarkably decreased in Keap1-KD mice compared to WT mice at 7 days after the nephrectomy (Figure 1f). Reduction of the preserved tubular areas in IRI kidneys at day 14 remained at day 42 in both WT and Keap1-KD mice (Figure 1g and h), suggesting that tubular damage at day 14 predicts the phenotypes in the late phase of injury. These data clearly indicate that Nrf2 hyperactivation preserves the renal function in the late phase of injury through preventing tubular damage.

Nrf2 protects renal tubules from IRI-induced oxidative stress in the early phase of injury
Hematoxylin-eosin staining of WT-IRI kidneys at day 1 revealed necrosis in the outer medulla (arrowheads in Figure 2a), where the most vulnerable renal tubular cells reside. The necrotic area was wider in Nrf2-KO-IRI kidneys and smaller in Keap1-KD-IRI kidneys than in WT-IRI kidneys, consistent with a previous report employing the Nrf2 activator CDDO (1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazolidine) in mice.15

Oxidative stress-mediated tubular damage at day 1 was analyzed by detecting the nuclear accumulation of an oxidative stress–associated DNA adduct, 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Supplementary Figure S2). Oxidative stress in the outer medulla was enhanced in Nrf2-KO-IRI kidneys but mitigated in Keap1-KD-IRI kidneys compared to WT-IRI kidneys (Figure 2b). An apoptosis marker, cleaved caspase-3, was also detected in the tubular cells of the outer medulla in WT-IRI kidneys at day 1 (Figure 2c). The cleaved caspase-3-positive areas spread toward the cortex in Nrf2-KO-IRI kidneys (arrowheads in Figure 2c), while Nrf2 activation by Keap1 knockdown appeared to ameliorate apoptosis. These results suggest that Nrf2 restricts the initial tubular injury in the outer medulla through mitigating IRI-induced oxidative stress.

In WT-IRI kidneys, the expression of Nrf2 target genes related to antioxidative stress response was induced until day 2, consistent with a previous report,22 and the induction was suppressed to basal levels until day 14 (Figure 3a). This transient induction in the early phase was undetectable in Nrf2-KO-IRI kidneys. In Keap1-KD-IRI kidneys, Nrf2 target genes were highly expressed before IRI treatment and further induced transiently (Figure 3a). Keap1-KD-IRI kidneys maintained high expression levels of Nrf2 target antioxidant genes throughout the observed period. Similarly, immunohistochemistry of NQO1 (NADPH: quinone acceptor oxidoreductase 1), an Nrf2-inducible factor, in the kidney sections at day 1 demonstrated that IRI slightly induces Nrf2 activity principally in renal tubules rather than in glomeruli and that this activity is further induced in Keap1-KD kidneys. In Nrf2-KO kidneys, basal NQO1 protein expression was very low, and no induction was observed after IRI treatment (Figure 3b). We thus consider that high expression levels and continuous enhancement of antioxidant genes ameliorate tubular damage in Keap1-KD-IRI kidneys.

Genetic activation of Nrf2 induces NADPH synthesis genes in injured kidneys
To identify other Nrf2 target genes related to kidney protection in addition to antioxidant genes, microarray analyses were conducted using normal kidneys and IRI kidneys at day 1
**Figure 1 | Genetic activation of Nrf2 ameliorates IRI-induced kidney damage in the late phase of injury.** (a) Elastica-Masson staining of ischemia-reperfusion injury (IRI) kidneys and contralateral uninjured kidneys from wild-type (WT), Nrf2 knockout (Nrf2-KO), and Keap1 knockdown (Keap1-KD) mice at 14 days after unilateral IRI (day 14). Bar = 200 μm. (b) Serial sections of WT-IRI kidneys at day 14 were stained with Elastica-Masson (left panels) and hematoxylin-eosin (right panels). In hematoxylin-eosin staining, immune cells (arrowheads) were identified as small round cells surrounding tubules that were weakly stained by Elastica-Masson (dotted yellow lines) but not around tubules stained red by Elastica-Masson (dotted green lines). Bar = 100 μm in upper panels and 30 μm in lower panels. (c) Serial sections of WT-IRI kidneys at day 14 were stained with Elastica-Masson (upper panels) (Continued)
from WT and Keap1-KD mice. The expression levels of known Nrf2 target genes\(^9,23\) were significantly higher in both normal and IRI kidneys of Keap1-KD mice than in WT mice (Figure 4a), consistent with the reverse transcription-quantitative PCR (RT-qPCR) data shown in Figure 3a. Notably, the expression of genes related to glycolysis and the tricarboxylic acid cycle were strongly suppressed by IRI in both genotypes of mice (Figure 4b and Supplementary Figure S3), suggesting the suppression of energy synthesis in the early phase of injury. The expression of genes encoding pentose phosphate pathway (PPP) enzymes was maintained in IRI kidneys of both genotypes of mice, and the expression of 4 genes (\(G6pd\), \(Pgdl\), \(Me1\), and \(Idh1\)) among the PPP enzyme genes was further induced in Keap1-KD mice (Figure 4b).

Nrf2 directly regulates the NADPH synthesis-related genes, including \(G6pd\), \(Pgdl\), \(Me1\), and \(Idh1\), and NADPH is essential for the recycling of oxidized glutathione. We found that the \(G6pd\) and \(Pgdl\) mRNA expression levels were transiently induced by injury in WT-IRI kidneys, similar to antioxidant genes, while no substantial changes were observed in the expression profiles of the \(Me1\) and \(Idh1\) genes (Figure 4c). The gene expression levels of PPP-related NADPH synthases were significantly enhanced in Keap1-KD-IRI kidneys, but those in Nrf2-KO-IRI kidneys were suppressed throughout the experimental period. These results suggest that Nrf2 protects tubular cells from IRI-induced oxidative stress through enhancing the synthesis of glutathione (GSH) and NADPH.

**Genetic activation of Nrf2 in injured kidneys increases the production of GSH and NADPH**

We then performed a distribution analysis of GSH by matrix-assisted laser desorption/ionization-imaging mass spectrometry and found that the signal intensity of GSH (m/z 306.07) was stronger in Keap1-KD mice than in WT mice, especially in the cortex under normal conditions (Figure 5a). This finding is the first direct demonstration by bioimaging that GSH production is significantly promoted by Nrf2 activation in mice.

Notably, the signal intensity for GSH in the cortex was robustly enhanced by IRI in Keap1-KD kidneys compared to that in WT-IRI kidneys (Figure 5b and c). In severely damaged areas in the outer medulla of WT-IRI kidneys, the GSH signal was remarkably low compared to that of Keap1-KD-IRI kidneys (dotted areas in Figure 5b). These data demonstrate that GSH synthesis is strongly enhanced in Keap1-KD-IRI kidneys by Nrf2 hyperactivation, especially in the cortex in which most tubules are preserved at day 1 (Supplementary Figure S4).

We also quantified the tissue concentrations of GSH and its precursors in normal kidneys and IRI kidneys at day 1 by CE-TOFMS (capillary electrophoresis time-of-flight mass spectrometry). While glutamate concentrations showed only marginal differences among the 4 types of kidneys, the concentrations of GSH, \(\gamma\)-glutamylcysteine and cysteine increased in Keap1-KD-IRI kidneys and to a lesser extent in normal kidneys of Keap1-KD mice (Figure 5d). We performed quantitative analysis of NADP\(^+\) and NADPH from these kidneys and found that while NADP\(^+\) levels were comparable, NADPH levels were significantly increased in Keap1-KD-IRI kidneys compared to WT-IRI kidneys (Figure 5e). These results suggest that GSH and NADPH synthesis is simultaneously promoted in the early phase of IRI by Nrf2 hyperactivation.

**Tubular Nrf2 is critical for the amelioration of IRI-induced tubular damage progression**

To identify cells responsible for renal protection by Nrf2 hyperactivation, a mouse line with the \(Keap1\) gene specifically deleted in the tubular cells of adult kidneys (tubule-specific Keap1 knockout [Keap1-TKO] mice) was analyzed. We first confirmed that the \(Keap1\) mRNA levels were dramatically reduced in Keap1-TKO kidneys compared to control kidneys (Figure 6a). At day 14, a greater number of preserved tubules was observed in IRI kidneys of Keap1-TKO mice compared to those of control mice, while no apparent morphological abnormalities were observed in the contralateral kidneys of Keap1-TKO mice (Figure 6b and c).

Significantly higher expression levels of Nrf2 target genes related to redox regulation and NADPH synthesis were detected in IRI kidneys of Keap1-TKO mice compared to those in IRI kidneys of control mice (Figure 6d). Therefore, we conclude that the cell-autonomous functions of Nrf2 in tubular epithelial cells contribute to protection against IRI-induced tubular damage through inducing antioxidant and NADPH synthesis genes. The preserved tubular area in Keap1-TKO-IRI kidneys at day 14 was slightly lower than that of Keap1-KD-IRI kidneys (28% and 33%, respectively; Figures 1d and 6b), suggesting that other kidney components, such as blood vessels, immune cells,26 or...
Figure 2 | Genetic activation of Nrf2 ameliorates IRI-induced kidney damage in the early phase of injury. Histological analyses of ischemia-reperfusion injury (IRI) kidneys and contralateral kidneys from wild-type (WT), Nrf2 knockout (Nrf2-KO), and Keap1 knockdown (Keap1-KD) mice at 1 day after unilateral IRI (day 1). (a) Hematoxylin-eosin staining of IRI kidneys. Necrotic areas (light pink, arrowheads) are shown. Bar = 200 μm. (b) Tissue sections of IRI kidneys and contralateral kidneys were stained with anti-8-OHdG antibody to detect IRI-induced oxidative stress. Specific signals (dark purple) were detected in nuclei (see high magnification images in Supplementary Figure S2). Arrowheads indicate black-stained protein casts likely derived from dead cells. Bar = 100 μm. (c) Immunohistochemical analyses of IRI kidneys with anti-cleaved caspase-3 antibody. Lower (upper panels) and higher (lower panels) magnification images are shown. Bar = 200 μm.
Figure 3 | Expression profiles of antioxidant genes in kidneys after IRI. (a) The expression of antioxidant genes in IRI kidneys of wild-type (WT), Nrf2 knockout (Nrf2-KO), and Keap1 knockdown (Keap1-KD) mice after ischemia-reperfusion injury (IRI) was analyzed by RT-qPCR. The data from contralateral kidneys of WT mice are also shown. The data are presented as the mean ± SE. **P < 0.01 compared with WT-IRI kidneys via Student’s t-test (n = 4–6 for each group). (b) NQO1 immunohistochemistry (brown stain) of IRI kidneys (upper panels) and contralateral kidneys (lower panels) from WT, Nrf2-KO, and Keap1-KD mice at day 14. Bar = 200 μm. (c) Higher magnification of the Keap1-KD-IRI kidney in b. Bar = 800 μm.
Figure 4 | Nrf2 activates genes for NADPH synthases in PPP in injured kidneys. (a,b) Heat map diagrams comparing gene expression levels among normal kidneys and ischemia-reperfusion injury (IRI) kidneys from wild-type (WT) and Keap1 knockdown (Keap1-KD) mice. Kidneys from 2 individual mice were analyzed in each group. IRI kidneys at 24 hours after IRI were used for the analyses. Representative Nrf2 target genes in a and genes related to energy metabolism in b are shown. (c) Expression levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH) synthesis genes in IRI kidneys of WT, Nrf2 knockout (Nrf2-KO), and Keap1-KD mice after IRI were analyzed by RT-qPCR. Data from the contralateral kidneys of WT mice are also shown. The data are presented as the mean ± SE. **P < 0.01 compared to WT-IRI kidneys via Student’s t-test (n = 4–6 for each group).
Figure 5 | Hyperactivation of Nrf2 enhances glutathione synthesis in the renal cortex of injured kidneys. (a-c) Matrix-assisted laser desorption/ionization-imaging mass spectrometry analysis of glutathione (GSH) in normal kidneys and ischemia-reperfusion injury (IRI) kidneys at day 1 with iMScope (Shimazu, Kyoto, Japan). Bright-field images are shown in upper panels, and damaged areas are indicated with yellow-dotted lines. Severely damaged areas were observed as dark gray areas in the outer medulla toward the cortex in wild-type (WT) IRI kidneys, but the damaged areas were limited in Keap1 knockdown (Keap1-KD) IRI kidneys. Bar = 500 μm. Analysis of GSH in normal kidneys of WT and Keap1-KD mice in a. Analysis of GSH in IRI kidneys of WT and Keap1-KD mice in b. Analysis of GSH in normal kidneys and IRI kidneys of Keap1-KD mice in c. (d,e) Capillary electrophoresis–mass spectrometry analysis in normal kidneys and IRI kidneys at day 1 from WT and Keap1-KD mice. Tissue concentrations of GSH and its precursors are shown in d, and relative changes in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADP⁺ are shown in e. Glutamate concentrations are statistically different, but the small differences (less than 5%) are probably not biologically meaningful. The data are presented as the mean ± SE. **P < 0.01 via Student’s t-test (n = 6).
renal fibroblasts, including renal erythropoietin-producing cells, may contribute to the Nrf2-mediated tubular protection.

Myeloid cells, including macrophages and neutrophils, are thought to be involved in tissue damage progression through the inflammatory response. To detect the infiltration of myeloid cells in injured kidneys, we exploited a tdTomato reporter mouse line (R26T mice) with a myeloid-specific deletion line, LysM-Cre (LysM-Cre:R26T mice). A massive increase in LysM-Cre–labeled myeloid cells was observed in the outer medulla of IRI kidneys at day 14 compared to contralateral kidneys (Figure 7a). Next, we generated myeloid-specific Keap1 knockout (Keap1-MKO) mice. Of note, myeloid-specific Keap1 deletion did not ameliorate tubular damage in IRI kidneys at day 14 (Figure 7b and c). These results demonstrate that Nrf2 hyperactivation in tubular cells, but not in myeloid cells, mainly contributes to tubular protection.

The G6PD gene is an essential target of Nrf2 for ROS scavenging in tubular cells

For further elucidation of the mechanisms underlying the tubular protective effects of Nrf2, the human tubular cell line HK-2 was incubated for 24 hours with menadione (also known as vitamin K3), which induces cell death as a result of ROS generation. Menadione killed HK-2 cells in a dose-dependent manner, and CDDO supplementation before menadione challenge significantly increased the cell survival rate (Figure 8a). Nrf2 target genes, including the G6PD gene, were induced immediately after CDDO supplementation, and the induction levels peaked at 12 to 24 hours after the supplementation (Figure 8b). CDDO also protected HK-2 cells from hypoxia-reoxygenation–induced cell death (Figure 8c). These data suggest that Nrf2 protects HK-2 from ROS-mediated cell death by inducing the expression of antioxidant and NADPH synthesis genes.

Transfection of HK-2 cells with siRNA for G6PD (siG6PD) resulted in a marked reduction of G6PD mRNA expression but did not affect the expression of another Nrf2 target gene (GCLM), indicating the specificity of siG6PD (Figure 8d). siRNA-mediated G6PD knockdown in HK-2 cells caused a robust increase in the dichlorofluorescein (DCF) signal, an indicator of intracellular ROS accumulation (Figure 8e). HK-2 cells transfected with a control siRNA (siNT) were

Figure 6 | Tubule-specific Keap1 knockout (Keap1-TKO) rescues kidneys from progressive tubular damage. (a) Keap1 mRNA expression in contralateral kidneys of control and Keap1-TKO mice at day 14. (b,c) Percentages of preserved tubular areas in the cortex of IRI kidneys of control and Keap1-TKO mice at day 14 were measured (b) in sections stained by Elastica-Masson (c). Bar = 200 μm. (d) Expression of Nrf2-target genes in both IRI kidneys and contralateral kidneys of control and Keap1-TKO mice at day 14. The data are presented as the mean ± SE. **P < 0.01 compared with kidneys from littermate control mice via Student’s t-test (n = 3–4 for each group).
marginally positive for this signal. Both in the absence and presence of CDDO, no obvious effects of G6PD knockdown were observed on menadione-induced cell death (Figure 8f), suggesting that the loss of G6PD was compensated by the other Nrf2-target genes to maintain cell survival. These results indicate the essential contribution of G6PD to ROS scavenging in tubular cells under physiological conditions.

**Short-term activation of Nrf2 in the early phase of injury is critical for ameliorating tubular damage progression**

We next examined whether post-injury pharmacological Nrf2 activation could elicit beneficial effects against kidney damage. Alternate-day administration of CDDO or vehicle was initiated on day 1 in WT mice, and the kidneys were analyzed at day 14 (protocol CC or VV, respectively; Figure 9a). Elastica-Masson staining revealed wider distribution of preserved tubules in IRI kidneys of protocol CC (33%) than in those of protocol VV (15%) (Figure 9a). These results demonstrate that post-injury hyperactivation of Nrf2 with CDDO prevents progressive tubular damage, consistent with the genetic activation of Nrf2 (34% in Figure 1d).

We then administered CDDO to WT mice only at the early time points (days 1, 3, and 5; protocol CV) or at the later time points (days 7, 9, 11, and 13; protocol VC). Histological analyses revealed wider distribution of the preserved tubules in IRI kidneys of protocol CV compared to those of protocols VC and VV at day 14 (Figure 9a). Protocol CV was as effective as protocol CC and Keap1-KD mice (Figures 1d and 9a). Although Nrf2 target gene expression was highly induced in IRI kidneys of protocol VC at day 14 (Figure 9b and
Supplementary Figure S5A), tubular damage was not ameliorated. As the induction of Nrf2 target gene expression was undetectable in IRI kidneys of protocol CV at day 14, we surmise that Nrf2 activation in the early phase of kidney injury is critical for tubular cell protection from damage progression.

Nrf2 activation is known to contribute to cellular proliferation; tubular repair and regeneration after AKI are also important for reducing tubular damage. However, the numbers of Ki67-positive proliferating cells were equal in the IRI kidneys of WT, Nrf2-KO and Keap1-KD mice at day 14 (Supplementary Figure S5B–D). Additionally, the expression profile of the gene for Ki67 (Mki67) was equivalent after IRI treatment among the kidneys of WT, Nrf2-KO and Keap1-KD mice (Supplementary Figure S5B–D). These data indicate that tubular cells were mainly protected from kidney injury by the Nrf2-dependent cytoprotective system but not by cellular proliferation and regeneration.

**DISCUSSION**

Because AKI is one of the major risk factors for CKD, prevention of AKI-CKD transition is critical for better patient outcomes. AKI is often caused by IRI, which induces a rapid burst of ROS that injure tubular cells. This study is
the first to demonstrate that hyperactivation of the antioxidant transcription factor Nrf2 in tubules prevents tubular damage progression through suppressing IRI-mediated oxidative stress in the early phase of kidney injury. Using chemical inducers, we also demonstrated that short-term Nrf2 activation in the early phase of injury is sufficient for preventing progressive tubular damage. As summarized in Figure 9c, we propose that Nrf2 hyperactivation in tubular cells induces both antioxidant and NADPH synthesis genes to enhance its cytoprotective ability and protects against progressive tubular damage after IRI. Thus, our findings provide important information for developing therapeutic strategies against AKI, especially for preventing subsequent development of CKD.

Figure 9 | Chemical activation of Nrf2 in the early phase of IRI protects against tubular damage progression. (a) The schematics of protocols (VV, CC, CV, and VC) for 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazolide (CDDO) administration. CDDO (30 μmol/kg body weight) or vehicle (sesame oil) was perorally administered to wild-type (WT) mice every 2 days for 14 days after unilateral ischemia-reperfusion injury (IRI). Percentages of preserved tubular areas in the cortex of IRI kidneys at day 14 were measured (upper right graph) in sections stained by Elastica-Masson (bottom). Bar = 200 μm. The data are presented as the mean ± SE. *P < 0.05, **P < 0.01 compared with IRI kidneys of protocol VV via Student’s t-test (n = 3–5 for each group). (b) Expression levels of Nrf2 target genes in IRI kidneys from differentially CDDO-treated mice at day 14. The mRNA expression levels in the contralateral kidneys of protocol VV are set as 1, and relative expression levels are shown. The data are presented as the mean ± SE. *P < 0.05, **P < 0.01 compared with IRI kidneys of protocol VV via Student’s t-test (n = 3–5 for each group). (c) Summary of this study. IRI causes oxidative stress-induced tubular injury and provokes tubular necrosis and apoptosis of the outer medulla in the early phase followed by progression of tubular damage in the cortex. Nrf2 is activated transiently in the early phase of IRI, but this activation does not last in the late phase. Importantly, hyperactivation of Nrf2 by Keap1 inhibition both genetically and chemically during only the early phase of IRI can enhance the expression of genes for the antioxidant response, as well as for detoxification (Nqo1, Srxn1, and Txnrd1), glutathione metabolism (Gclm and Gstm1), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) synthesis (G6pd and Pgd), thereby preventing the expansion of tubular damage of the cortex in the late phase and eventually preventing the transition to CKD.
demonstrates that myeloid-specific Nrf2-dependent kidney protection. Although this study induction of proliferative cues is not primarily involved in of the highest priority therapeutic targets for the hyper-
1 day after renal IRI treatment but did not affect cellular damage. Additionally, Nrf2 hyperactivation mitigated both IRI-induced oxidative stress and cell death in tubular cells at 1 day after renal IRI treatment but did not affect cellular proliferation. These results demonstrate that tubules are one of the highest priority therapeutic targets for the hyper-activation of Nrf2 among kidney components via the suppression of IRI-induced oxidative stress and that the induction of proliferative cues is not primarily involved in Nrf2-dependent kidney protection. Although this study demonstrates that myeloid-specific Nrf2 activation is ineffective for renal protection, T-cell-specific Nrf2 activation has been reported to ameliorate kidney injury by suppressing the inflammatory response, suggesting that T lymphocytes, but not myeloid cells, are also therapeutic targets for kidney protection.

PPP-associated genes and antioxidant genes were highly induced in Nrf2-activated kidneys at 1 day after IRI treatment. In addition to nucleotide synthesis, PPP plays an important role in the synthesis of NADPH, which is essential for the reduction and reactivation of antioxidant enzymes coded by the Nqo1, Hmox1, TxnrD1 and Gr1 genes. Notably, these genes are activated by Nrf2 in an oxidative stress-inducible manner. Indeed, the data from matrix-assisted laser desorption/ionization-imaging mass spectrometry and capillary electrophoresis–mass spectrometry showed that Nrf2 hyperactivation promotes the production of GSH and NADPH in injured kidneys. These findings are consistent with a recent report that an Nrf2 activator induces both GSH and NADPH production in mouse kidneys. As the knockdown experiment showed the essential role of the PPP-associated NADPH synthase G6PD in scavenging intracellular ROS, it is plausible to consider that G6PD contributes to renal tubular protection against IRI-induced oxidative stress through NADPH production. In fact, G6PD-deficient patients and mice exhibit susceptibility to oxidative stress.

Pretreatment with chemical inducers for Nrf2 has been shown to result in protective effects in AKI mouse models. Our extensive analyses demonstrated that Nrf2 hyperactivation by CDDO during the early time points after renal IRI treatment in mice, but not during the late phase, most efficiently protects tubules. Therefore, we surmise that Nrf2 inducers are ideal and effective therapeutic agents for critically ill patients with unpredictable AKI due to hemodynamic instability, such as sepsis, hemorrhage or heart failure, in addition to predictable AKI due to renal transplantation or cardiovascular surgery.

In summary, this study is the first to identify that endogenous Nrf2 activation is insufficient and that genetic or pharmacological hyperactivation of Nrf2 in tubular cells is necessary to maximize its cytoprotective function against progressive tubular injury. Thus, pharmacological Nrf2 activation is an attractive therapeutic strategy for the prevention of IRI-mediated progressive kidney injury, which may ultimately prevent the AKI-CKD transition.

MATERIALS AND METHODS
A detailed Supplementary Methods section and Supplementary Tables S1 and S2 are available in the online-only Supplementary material.

Mice
Nrf2-KO (Nrf2<sup>fl/fl</sup> genotype) and Keap1-KD (Keap1<sup>hd/hd</sup> genotype) mice were previously generated in-house. A transgene expressing a reverse tetracycline-controlled trans-activator (rtTA) under the tubule-specific flox8 gene promoter was integrated into Keap1 conditional KO mice with an rtTA-inducible Cre recombinase transgene to generate renal tubule-specific Keap1-TKO (Keap1<sup>floxflox;</sup> Tg<sup>flox–rtT</sup>A: Tg<sup>Teto–Cre</sup> genotype) mice; the 2-month-old mice were treated with doxycycline (Sigma-Aldrich, St. Louis, MO) in the drinking water (0.4 g doxycycline per 100 ml solution containing 5% sucrose) for 6 weeks before analyses with littermate control mice treated with doxycycline (control mice, Keap1-TKO mice without Tg<sup>flox–rtT</sup>A: Tg<sup>Teto–Cre</sup>). Keap1-MKO (Keap1<sup>floxflox;</sup> Lysmd<sup>C</sup>Cre<sup>+</sup> genotype) mice were generated by crossbreeding Keap1<sup>floxflox;</sup> mice with LysM-Cre myeloid-specific Cre-expressing mice, in which Cre cDNA was inserted into the Lysmd<sup>2</sup> gene. These mice were analyzed with littermate control mice bearing the Keap1<sup>floxflox;</sup> Lysmd<sup>C</sup>Cre<sup>Lysmd2<sup>+/+</sup> or Keap1<sup>+/+</sup>:Lysmd2<sup>+/+</sup></sup> genotype. To detect LysM-Cre-labeled myeloid cells in injured kidneys, a tdTomato reporter mouse line for Cre excision (R26T mouse, Rosa26<sup>cre-loxp-STOP-loxp-tdTomato+</sup> genotype; Jackson Laboratory, Bar Harbor, ME) was crossed with LysM-Cre mice (LysM-Cre:R26T mice). All mice were in a C57BL/6J background and were analyzed at 2 to 4 months of age. CDDO (Mochida Pharmaceuticals, Tokyo, Japan) in sesame oil (30 μmol/kg of body weight; Wako Pure Chemical Industries, Osaka, Japan) was orally administered every 2 days starting from 1 day after IRI (days 1, 3, 5, 7, 9, 11, and 13). The sequences for the primers used for genotyping are listed in Supplementary Table S1. The mice were maintained in a specific pathogen-free facility. All animal experiments were approved by the Animal Care Committee at Tohoku University.

Unilateral IRI and unilateral nephrectomy
Mice were anesthetized by i.p. injection of pentobarbital (75 mg/kg; Wako Pure Chemical Industries, Osaka, Japan) and warmed on a heating pad (38°C) to maintain their body temperature during the procedure, and an abdominal incision was made. For unilateral IRI, the left renal vein and artery were clamped for 45 min by a plastic disposable vascular clip (Bear Medic Corporation, Tokyo, Japan), and then the abdominal wound was sutured immediately after releasing the clip. The right renal vessels were not clamped, in order to compare the left damaged kidneys (IRI kidneys) with the right untreated kidneys (contralateral kidneys). For unilateral nephrectomy, after ligation of the renal vein, artery and ureteral duct, the contralateral kidney was removed to analyze the renal function of the IRI kidney alone.

Gene expression analysis
Total RNA samples (3 μg for kidneys and 1 μg for culture cells) prepared by Isogen (Nippon Gene, Tokyo, Japan) were reverse-transcribed using random hexamers and a Superscript III polymerase kit (Invitrogen, Carlsbad, CA). RT-qPCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with Thunderbird SYBR qPCR Mix or Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan). The sequences of the primers...
used are listed in Supplementary Table S2. Gene expression levels were calculated based on the threshold cycle values (Ct) and on the efficiency of each primer set and standardized to Ct values for 18S rRNA expression levels. For microarray analyses, total RNA was purified from normal kidneys or IRI kidneys and hybridized with mouse gene expression microarrays (4 × 44 K, Agilent Technologies, Santa Clara, CA; n = 2 for each experimental group) following the manufacturer’s procedure. The data were analyzed using Gene Spring software (Agilent Technologies) and submitted to Gene Expression Omnibus (accession no. GSE71647).

Analysis of renal functions
Blood samples were obtained from the buccal artery of mice. After the blood samples were centrifuged, plasma creatinine levels were measured by DRI-CHEM 7000V (FUJIFILM, Tokyo, Japan) using a DRI-CHEM SLIDE (CRE-P III).

Data analysis
The data are shown as the mean ± SE. Comparisons between groups were conducted using one-way ANOVA or unpaired t-tests as appropriate. The data were considered statistically significant at P < 0.05.

DISCLOSURE
All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods.
Figure S1. Detection of damaged and preserved tubular cells by Elastica-Masson staining of kidney sections. (A) Sections of normal kidneys (day 0) and IRI kidneys (days 1, 2, 3, 7, and 14) from WT mice were stained with Elastica-Masson. High-magnification images are shown in the right panels. Bar = 200 µm. (B) Preserved tubular areas in the cortex are measured using NIH Image software. An Elastica-Masson-stained section of WT-IRI kidney at day 14 is shown as an example. (C) Time-course analysis of quantification of preserved tubular areas (red areas in a) in the cortex of WT-IRI kidneys from day 0 to day 14. The data are presented as the mean ± SE. **P < 0.05, ***P < 0.01 compared to normal kidneys of WT mice via Student’s t-test (n = 3–5 for each group). (D) Morphologic changes of tubular cells in the cortex as determined by electron microscopy. Asterisks indicate tubular cells in both IRI kidneys at day 14 and contralateral kidneys. Bar = 2 µm.

Figure S2. Histological detection of tubular cell injury. Strong nuclear staining with anti-8-OHdG antibody in tubules was observed in a WT-IRI kidney section at day 1, but not in that of contralateral kidney. These data suggest that IRI induces oxidative stress in tubules at day 1. Bar = 100 µm.

Figure S3. Heat map diagram showing the expression profiles of all glycolytic genes. Normal kidneys and IRI kidneys from WT and Keap1-KD mice at day 1 are shown (n = 2 for each group).

Figure S4. The cortices of IRI kidneys at day 1 were morphologically preserved. Elastica-Masson staining of IRI kidneys from WT, Nrf2-KO and Keap1-KD mice at day 1. Bar = 100 µm.

Figure S5. Nrf2 protects tubules from progressive tubular damage in a cell proliferation-independent manner. (A) Analyses of NQO1 immunohistochemistry in IRI kidneys (upper panels) and contralateral kidneys (lower panels) from mice with different CDDO administration protocols (protocol VV, CC, VC, and CV). Bar = 200 µm. (B) Anti-Ki67 immunohistochemistry (brown signals) of IRI kidney sections from WT, Nrf2-KO, and Keap1-KD mice at day 14 is shown at lower (upper panels) and higher (lower panels) magnifications. Bars = 100 µm. (C) Nuclei positive for Ki67 staining were counted in IRI kidney sections from WT, Nrf2-KO, and Keap1-KD mice at day 14. Ki67-positive cells were counted in 4 random areas of a mouse kidney, and data from 3–5 mice in each genotype are presented as the mean ± SE. (D) Expression levels of the Mki67 gene in IRI kidneys from WT, Nrf2-KO and Keap1-KD mice after IRI were analyzed by RT-qPCR. Data from the contralateral kidneys of WT mice are also shown. The data are presented as the mean ± SE. **P < 0.01 compared to WT-IRI kidneys via Student’s t-test (n = 4–6 for each group).

Table S1. Oligonucleotide sequences of the primers used for genotyping PCR in this study.

Table S2. Oligonucleotide sequences of the primers used for qPCR in this study.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES