NFAT5 Is Protective Against Ischemic Acute Kidney Injury

Shoujin Hao, Lars Bellner, Hong Zhao, Brian B. Ratliff, Zbigniew Darzynkiewicz, Carlos P. Vio, Nicholas R. Ferreri

Abstract—NFAT5 is a transcription factor that protects the kidney from hypertonic stress and also is activated by hypoxia. We hypothesized that NFAT5 mitigates the extent of renal damage induced by ischemia–reperfusion injury (IRI). Mice were subjected to IRI by unilateral clamping of the left renal pedicle for 30 minutes followed by reperfusion. After 3 hours of reperfusion, the level of NFAT5 mRNA was similar in contralateral and clamped kidneys. However, after 48 hours, NFAT5 mRNA accumulation increased ≈3-fold in both outer medulla and medullary thick ascending limb tubules. NFAT1 levels were elevated at 3 hours but did not increase further at 48 hours. Mice were then pretreated for 72 hours with an intrarenal injection of a lentivirus short-hairpin RNA construct to silence NFAT5 (enhanced green fluorescent protein-U6-N5-ex8) or a control vector (enhanced green fluorescent protein-U6) before induction of IRI. Neutrophil gelatinase–associated lipocalin and kidney ischemia molecule-1 mRNA levels increased after IRI and further increased after knockdown of NFAT5, suggesting that silencing of NFAT5 exacerbates renal damage during IRI. In contrast, silencing of NFAT1 had no effect on the levels of neutrophil gelatinase–associated lipocalin or kidney ischemia molecule-1 mRNA. Hematoxylin and eosin staining revealed patchy denudation of renal epithelial cells and tubular dilation when NFAT5 was silenced. The number of TUNEL-positive cells in the outer and inner medulla of the clamped kidney increased nearly 2-fold after knockdown of NFAT5 and was associated with an increase in the number of caspase-3–positive cells. Collectively, the data suggest that NFAT5 is part of a protective mechanism that limits renal damage induced by IRI.

Key Words: acute kidney injury • NFAT transcription factor 5 • reperfusion injury

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Received September 25, 2013; first decision October 16, 2013; revision accepted November 14, 2013.

From the Departments of Pharmacology (S.H., L.B., N.R.F.), Medicine and Physiology (B.B.R.), and Pathology (H.Z., Z.D.), New York Medical College, Valhalla; and Department of Physiology, Pontificia Universidad Católica de Chile, Santiago, Chile (C.P.V.).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.02476/-/DC1.

Correspondence to Nicholas R. Ferreri, Department of Pharmacology, New York Medical College, Valhalla, NY 10595. E-mail nick_ferreri@nymc.edu

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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.113.02476
Antibodies
The anti-NFAT5 antibody (Santa Cruz) was used at a 1:1000 dilution, and anti-enhanced green fluorescent protein (EGFP) antibody (Abcam) was used at a 1:10000 dilution.

Plasmid Constructs and Virus Preparation
The NFAT5-dominant negative expression plasmid was generated as previously described.12 The inhibitory construct for NFAT5 or NFAT1 was designed using a short-hairpin RNA—expressing construct targeting exon 8 of murine NFAT5 (U6-N5-ex8) or NFAT1 (U6-N1-ex8), as described previously.10,12 Subcloning of EGFP, U6-N5-ex8, or U6-N1-ex8 into a pLKO.1 vector and cotransfecting HEK293-T cells with pLKO.1 was performed to generate lentivirus encoding EGFP, U6-N5-ex8, or U6-N1-ex8.

Lentivirus Preparation and Administration
Generation of lentiviral supernatants was performed as previously described using psPAX2, pMD2.G (Addgene), and pLKO.1 or psiLV plasmids.13 In anesthetized mice, a 31-gauge needle was inserted at the lower pole of both kidneys parallel to the long axis and was carefully pushed toward the upper pole. As the needle was slowly removed, 50 μL filter-purified lentivirus (EGFP, U6-N5-ex8, or U6-N1-ex8) was injected. Lentiviral-mediated EGFP protein expression in kidney parenchyma was robust after 72 hours.13

Isolation of mTAL Tubules and Cells
mTAL tubules and cells (90%–95% purity) were isolated from mice as previously described12 and as detailed in the online-only Data supplement.

Transient Transfection of mTAL Cells
mTAL cells were cultured to 70% to 80% confluence in 6-well plates on membrane inserts (BD Biosciences) and transfected using Lipofectamine 2000 as previously described.12

Model of Renal IRI
The left renal pedicle was clamped for 30 minutes with microvascular clips (FE 723 K, Aesculap) to induce ischemia, which was verified by the change of renal color. Clamps were not applied in the sham group. After removal of the clamp, mice were euthanized at 3 and 48 hours to examine the change of renal color. Clamps were not applied in the sham group.

Isolation of Total RNA and Amplification of cDNA Fragments/Quantitative Real-Time Polymerase Chain Reaction
Total RNA was isolated from medulla, mTAL tubules, and mTAL cells as previously described; see online-only Data supplement for quantitative real-time polymerase chain reaction analysis.13

Western Blot Analysis
Solubilized samples were heated at 60°C in loading buffer and protein concentration determined with a Bio-Rad protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, blocked, and probed at 4°C overnight with primary antibodies. After incubation with horseradish peroxidase–conjugated secondary antibodies (Amersham), proteins were detected by enhanced chemiluminescence (Amersham).

Immunohistochemistry Analysis
Kidneys were perfusion fixed in situ with 4% paraformaldehyde, fixed in 4% neutral-buffered paraformaldehyde overnight, and then embedded in paraffin. Serial sections (3–5 μm) were stained with hematoxylin and eosin or antibodies against EGFP and caspase-3.

Statistics
All data are presented as mean±SE. Statistical analysis was performed using a 1-way ANOVA followed by Tukey multiple comparisons or unpaired t test as appropriate. Differences with P<0.05 were considered statistically significant.

Results

Figure 1, A to C, Validation of control and silencing lentivirus constructs in vivo. A lentivirus construct targeting exon 8 of NFAT5 (EGFP-U6-N5-ex8) was designed to silence NFAT5 in vivo. The construct was injected directly into the kidney and the EGFP transgene was used as a marker to verify expression 72 hours after injection of the construct. CTRL indicates control; EGFP, enhanced green fluorescent protein; TAL, thick ascending limb.

Construction and Validation of a Lentivirus Construct to Silence NFAT5 In Vivo
A short-hairpin RNA construct targeting exon 8 of NFAT510 was used to prepare a novel lentivirus construct (EGFP-U6-N5-ex8) to silence NFAT5 expression in vivo. Kidneys were harvested from mice given an intrarenal injection of EGFP-U6 (control) or EGFP-U6-N5-ex8 containing a cytomegalovirus promoter to drive expression of the EGFP gene. Western blot analysis showed robust expression of EGFP in mTAL tubules obtained from mice 72 hours after administration of lentivirus constructs (Figure 1A). EGFP expression also was detected by immunofluorescence microscopy in OM and TAL tubules from kidneys injected with lentivirus constructs but not PBS (control; Figure 1B and 1C, respectively). Furthermore, endogenous NFAT5 mRNA accumulation was reduced by ≈70% (Figure 2A and 2B). NFAT5 protein expression also was markedly inhibited in OM isolated from mice.
injected with EGFP-U6-N5-ex8 but not the control construct (EGFP-U6; Figure 2C). In addition, knockdown of NFAT5 prevented the increase in Na\textsuperscript{+}/myo-inositol cotransporter, a downstream target of NFAT5 that was induced when mTAL cells were exposed to media made hypertonic by addition of NaCl (400 mOsm/kg H\textsubscript{2}O; Figure 2D). Collectively, these data show that the silencing vector blocks NFAT5 transcriptional activity and is effective in vivo.

**Expression of NFAT5 and NFAT1 in OM and mTAL Tubules After IRI**

Kidneys were recovered 3 and 48 hours after mice were subjected to 30 minutes of IRI induced by unilateral clamping of the left renal pedicle, and NFAT isoform mRNA levels were determined by quantitative real-time polymerase chain reaction analysis in OM isolated from the clamped kidney or kidney of a sham-operated mouse. After 3 hours of reperfusion, the relative abundance of NFAT5 mRNA was similar in OM from sham and ischemic kidneys, whereas NFAT1 mRNA increased (Figure 3A and 3B). However, after 48 hours of reperfusion, NFAT5 mRNA accumulation increased ≈3-fold in both mTAL tubules and OM, whereas NFAT1 mRNA accumulation did not increase further (Figure 3A–3D). The increase in NFAT5 in both mTAL tubules and OM was inhibited in mice that were pretreated with the lentivirus construct, U6-N5-ex8, designed to silence NFAT5 (Figure 3C and 3D). Together, these data suggest that NFAT5 and NFAT1 are differentially regulated during IRI, and the targeting vector efficiently silences NFAT5 during IRI.

**Effect of NFAT5 Silencing on Biomarkers of Renal Damage During IRI**

Kidney ischemia molecule (Kim)-1 and neutrophil gelatinase–associated lipocalin (NGAL) in the urine originate from damaged renal tubular epithelial cells and correlate well with their respective mRNA levels in the kidney.\textsuperscript{5,14,15} Because NGAL is a biomarker that anticipates the diagnosis of acute kidney injury and is expressed specifically in distal tubular segments injured by ischemia,\textsuperscript{14,15} the effects of NFAT5 silencing on NGAL mRNA levels was determined in OM after 48 hours of reperfusion. A marked increase in NGAL and Kim-1 mRNA levels were observed in OM from clipped kidneys compared with kidneys from sham-operated mice (Figure 4A and 4B). Silencing of NFAT5 in mice pretreated with U6-N5-ex8 further increased the mRNA levels of NGAL and Kim-1 (Figure 4A and 4B). In contrast, although NFAT1 mRNA accumulation remained elevated 48 hours after reperfusion in OM, silencing of NFAT1 (U6-N1-ex8) did not affect NGAL or Kim-1 mRNA levels (Figure 4C and 4D). The data suggest that inhibition of NFAT5, but not NFAT1, worsens injury to nephron segments after IRI.

**Silencing of NFAT5 Exacerbates Renal Damage Induced by IRI**

Intensified damage to both the proximal and distal tubule may have occurred when NFAT5 was silenced before initiation of renal ischemia, and high expression of NFAT5 in inner medullary (IM) collecting ducts may also make this region of the nephron susceptible to damage. Accordingly, we evaluated the effect of silencing NFAT5 on renal damage by evaluating sections from OM and IM that were stained with hematoxylin and eosin after 30 minutes of unilateral IRI and 48 hours of reperfusion. Silencing of NFAT5 aggravated damage in OM and IM, whereas injection of the control vector had no effect (Figure 5). Both regions of the medulla displayed patchy loss (denudation) of renal tubular cells and flattened tubular epithelium resulting in tubular dilation when NFAT5 was silenced, suggesting that NFAT5 is part of a mechanism that diminishes damage elicited by IRI.

**TUNEL Analysis of Apoptosis During IRI**

The nature of tubular destruction following silencing of NFAT5 was assessed to identify pathways that may be linked to this transcription factor. Because apoptosis is part of the profile of damage induced by IRI, we examined the effect of silencing NFAT5 on the frequency of TUNEL-positive cells. There were few TUNEL-positive cells in OM or IM of IRI mice pretreated with control lentivirus (IRI+U6), in concert with the relatively mild protocol selected for the present study (unilateral 30-minute occlusion; Figure 6). However, silencing of NFAT5 was associated with a distinct increase in the frequency of TUNEL-positive cells in the OM (Figure 6). The renal OM is sensitive to damage because of hypoxia. However, because no information is available regarding the function of NFAT5 in IRI, we also measured apoptosis in the cortex and IM. Analysis by laser scanning cytometry showed...
that the frequency of TUNEL-positive cells in OM and IM increased ≈2- to 3-fold after silencing of NFAT5 (Figure 7). In contrast, silencing of NFAT5 did not result in damage to the cortex. These findings suggest that apoptosis may be part of the mechanism that contributes to IRI-induced renal medullary damage in the absence of NFAT5.

**Knockdown of NFAT5 Increases Caspase-3 Expression**

Because both caspase-dependent and caspase-independent pathways induce apoptosis, the effect of silencing NFAT5 on caspase-3 expression was determined in sections of OM. Few caspase-3-positive cells were observed after IRI and 48 hours of reperfusion in mice with an intact NFAT5 response, whereas silencing of NFAT5 increased the frequency of caspase-3–positive cells (Figure 8). The results suggest that knockdown of NFAT5 predisposes mice subjected to IRI to increased apoptosis by a mechanism involving caspase-3 activation.

**Discussion**

We demonstrated that silencing of NFAT5 markedly increased renal damage in a model of IRI. NFAT5 mRNA levels increased in response to 30 minutes of unilateral IRI and 48 hours of reperfusion. Silencing of NFAT5 increased NGAL and Kim-1, biomarkers of renal damage in IRI. In contrast, knockdown of NFAT1, which also was elevated 48 hours after reperfusion, did not affect NGAL and Kim-1 levels. Extensive damage that included denudation of tubular epithelial cells and tubular dilatation was observed in OM and IM, but not in the cortex, of mice in which NFAT5 was silenced before initiation of IRI. Moreover, silencing NFAT5 increased the number of TUNEL-positive cells in OM and IM but not the...
cortex, and the increase in apoptotic cells was associated with an increase in caspase-3 expression. Collectively, the data suggest that NFAT5 acts as a protective transcription factor in IRI by diminishing the extent of renal injury via a mechanism that includes suppression of caspase–3–dependent apoptosis.

Recovery from acute kidney injury includes restoration of glomerular filtration rate and tubular repair but may lead to impaired renal hemodynamic and natriuretic responses that contribute to the development of salt-sensitive hypertension and chronic kidney disease. Elucidation of adaptive mechanisms that lessen renal damage induced by ischemic injury will establish a framework that provides options for new therapeutic approaches. The failure to increase NFAT5 during an early (3 hours) stage of reperfusion injury coupled with its increase during the reparative phase (48 hours) supports the notion that this transcription factor may be part of a protective mechanism that attenuates damage to hypoxic injury. In general, NFAT5 exhibits several functions that are renal protective, including the critical roles it plays as part of the adaptive response to hypertonic stress and during renal development.8,16 For instance, deletion of NFAT5 in mice causes embryonic and perinatal lethality associated with dramatic effects on the architecture of the renal medulla, whereas the cortex was relatively normal.16 Increased apoptosis also was primarily observed in the medulla. These findings are consistent with the increased damage and apoptosis observed in OM and IM from mice that lacked an adequate NFAT5 response when subjected to IRI in the present study.

Our data are consistent with studies showing that 30 minutes of unilateral IRI produces limited damage to S3 segment of the proximal tubules.1 In general, longer and more severe ischemia is required to elicit significant distal nephron injury. The capacity of the distal tubule to use anaerobic glycolysis under conditions of diminishing oxygen availability also contributes to the resistance of the TAL to damage induced by hypoxic injury, providing that energy expenditure for ion transport is limited. Indeed, transport function is a major determinant of TAL injury in the context of limited oxygen availability, and damage to the TAL is observed when oxygen consumption for ion transport is exaggerated.11,17,18 We previously showed that NFAT5 is part of a mechanism in the TAL that increases tumor necrosis factor-α production, which acts as an endogenous inhibitor of Na+-K+-2Cl− cotransporter activity.19 Given the delicate balance between oxygen supply and demand with respect to cellular viability, it is tempting to speculate that attenuation of ion transport pathways is part of the mechanism by which NFAT5 is protective during IRI. The contribution of hypertonicity to the increasing damage in IRI following NFAT5 knockdown has not yet been addressed directly. However, preliminary experiments showed that silencing of NFAT5 within the time frame of the ischemia–reperfusion protocol used in the present study did not affect water intake or urine volume (Hao, unpublished data, 2013). In addition, the increase in NFAT5 in response to hypoxia in inner medullary collecting duct was similar under isotonic and hypertonic conditions.9

Renal ischemic damage may be induced by several different mechanisms. For instance, TAL segment targeting of the von Hippel-Lindau protein, which mediates degradation of HIF, limited IRI-induced damage in the proximal tubule as well as the TAL without a corresponding reduction in inflammatory cell infiltration.7 Alternatively, targeted apoptosis in the TAL is sufficient to induce severe acute kidney injury, and cross-talk between S3 segments of the proximal tubule and TAL via Tamm-Horsfall glycoprotein protects the kidney against ischemia by inhibiting inflammatory signaling between these adjacent nephron segments.20,21 Such mechanisms are consistent with the finding that inflammatory cell recruitment need not be affected to improve
renal damage. HIF is induced rapidly after IRI and then diminishes by 48 hours. In contrast, NFAT5 expression was unchanged 3 hours after IRI but increased at 48 hours. Because both these transcription factors lessen renal damage associated with IRI, the temporal distinction suggests that HIF and NFAT5 may contribute to different aspects of renal protection during IRI. In addition, von Hippel-Lindau protein deletion in the TAL did not affect the number of apoptotic cells unlike silencing of NFAT5, which increased apoptosis in the present study. Thus, it is possible that the detrimental effects of silencing NFAT5 may be unrelated to HIF mechanisms, which supports in vitro data showing that HIF and NFAT5 are regulated independently.

Cell death programs including apoptosis, autophagy-associated cell death, and necrosis are initiated in response to IRI and are invoked or suppressed to different degrees by various molecules. For instance, although the renal protective effect of Tamm-Horsfall glycoprotein involved suppression of the inflammatory response, there did not seem to be an effect on apoptosis, suggesting that Tamm-Horsfall glycoprotein may inhibit processes related to necrosis. There also was no difference in the number of apoptotic cells in wild-type and von Hippel-Lindau protein–deficient mice. In contrast, apoptosis was more severe in renalsease-deficient mice and in the present study after NFAT5 silencing in mice subjected to IRI. Caspases are thought to play an important role in apoptosis as well as necrotic cell death. However, a recent study showed that cleaved caspase-3 was absent during the course of IRI and suggested this pathway may be of relatively minor importance in IRI. The increase in caspase-3 expression following NFAT5 silencing suggests that an NFAT5-dependent mechanism offsets renal damage induced by apoptosis in renal epithelial cells by suppressing caspase-3–dependent apoptosis.

The precise signals that induce NFAT5 during IRI are not yet defined and may include hypoxia as well as a change in the osmolality of the medulla because of altered expression of renal transporters. Nevertheless, NFAT5 seems to function as an upstream regulator of molecules and signaling pathways that integrate mechanisms directly related to tubular cell survival in the context of IRI.

Perspectives

Acute kidney injury results in rapid loss of renal function in association with death of tubular epithelial cells. This damage occurs despite adaptive changes within the kidney that attempt to minimize injury induced by hypoxia at the cellular level. Hypertension and other conditions such as diabetes mellitus may potentiate the ongoing impairment and accelerate the progression to chronic kidney disease. We demonstrated that induction of NFAT5 during the reparative phase of renal IRI is part of a mechanism that limits the extent of kidney injury induced by hypoxia. Moreover, the...
The present study suggests that NFAT5 may function as a molecular switch that directs renal epithelial cell fate toward survival after ischemic injury. Future studies will evaluate the molecular mechanisms that trigger induction of caspase-3–dependent apoptosis observed following silencing of NFAT5.

**Sources of Funding**

This work was supported by National Institutes of Health grant HL34300, American Heart Association grant 12GRNT12060350, Fondecyt 1130741, and PGB 12–2007 CARE UC-SQM.

**References**


**Novelty and Significance**

**What Is New?**

- This is the first report to show a protective effect of NFAT5 in an in vivo model of hypoxia-induced renal damage.
- When NFAT5 was silenced in vivo, increased damage was observed in renal epithelial cells in the outer and inner medulla.
- The protective effects of NFAT5 are related to suppression of caspase-3–dependent apoptosis.

**What Is Relevant?**

- Renal functional changes during acute kidney injury include altered tubuloglomerular feedback because sodium reabsorption in the proximal tubule is decreased, thereby causing afferent arteriole constriction and a decrease in glomerular filtration rate—a hallmark feature of acute kidney injury.
- Recovery from acute kidney injury includes restoration of glomerular filtration rate and tubular repair but may be associated with impaired renal hemodynamic and natriuretic responses that contribute to the development of salt-sensitive hypertension and chronic kidney disease.
- Understanding the mechanisms that limit excessive damage in IRI may reveal novel targets that can be exploited to exert a positive influence on the repair, regeneration, and remodeling of the injured tubule.

**Summary**

NFAT5 is activated during hypoxic stress to the kidney as part of a protective mechanism that limits apoptotic cell death in the renal medulla. Subversion of NFAT5-dependent protective mechanisms may promote the transition of acute kidney injury to chronic kidney disease and the development of hypertension.
NFAT5 Is Protective Against Ischemic Acute Kidney Injury
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Hypertension. 2014;63:e46-e52; originally published online December 30, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.02476

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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ONLINE SUPPLEMENTAL METHODS
NFAT5 is protective against ischemic acute kidney injury

Shoujin Hao¹, Lars Bellner¹, Hong Zhao³, Brian B. Ratliff², Zbigniew Darzynkiewicz³, Carlos P. Vio⁴, and Nicholas R. Ferreri¹

Departments of Pharmacology¹, Medicine and Physiology², Pathology³, New York Medical College, Valhalla, NY 10595 and ⁴Pontificia Universidad Catolica de Chile, Santiago, Chile
This work was supported by NIH grant HL34300, American Heart Association grant 12GRNT12060350, Fondecyt 1130741, and PGB 12-2007 CARE UC-SQM.

To whom correspondence and reprint requests should be addressed:
Dr. Nicholas R. Ferreri
Dept. of Pharmacology
New York Medical College
Valhalla, NY 10595
Phone: 914-594-4079
FAX: 914-347-4956
E-mail: nick_ferreri@nymc.edu
Running title: NFAT5 limits IRI damage
Animals-

Male C57BL/6J mice (8–12 wk) purchased from Jackson Laboratory were maintained on standard diet and given tap water *ad libitum*. Experimental procedures were conducted in accordance with institutional and international guidelines for the welfare of animals (animal welfare assurance number A3362-01, Office of Laboratory Animal Welfare, PHS, NIH).

Chemicals and reagents-

The anti-NFAT5 antibody was purchased from Santa Cruz Biotechnology (California) and used at 1:1,000 dilution, the antibody for EGFP (Abcam, cat. ab290) (Cambridge, UK) was used at a 1:10,000 dilution. Tissue culture media was obtained from Life Technologies (Grand Island, NY).

Isolation of mTAL tubules and cells-
mTAL tubules and cells (90-95% purity) were isolated from mice as previously described. Briefly, male C57BL/6J mice were anesthetized with an intraperitoneal injection of pentobarbital (0.065 mg/g body wt). The kidneys were perfused with sterile 0.9% saline and outer medulla was excised, minced with a sterile blade, and incubated for 10 min at 37 C in a 0.01% collagenase solution gassed with 95% oxygen. The suspension was sedimented on ice, mixed with Hanks’ balanced salt solution (HBSS) containing 2% BSA, and the supernatant containing the crude suspension of tubules collected. The remaining undigested tissue and combined supernatants were centrifuged for 10 min, resuspended in HBSS, and filtered though a 53 µm nylon mesh membrane (Fisher Scientific, Springfield, NJ). The filtered solution was discarded and tubules retained on the mesh were resuspended in HBSS and used in experiments or to establish primary cultures of mouse mTAL cells.

Plasmid constructs and virus preparation-

A NFAT5-dominant negative (NFAT5-DN) expression plasmid was generated by cloning a NFAT5 cDNA fragment containing the NFAT5 DNA binding domain (DBD) into pcDNA3.1 vector (Invitrogen); a cDNA (nucleotides 1074-1694) encoding amino acids 175-471, NM_018823.1) was used in this study. The cDNA was amplified from the pBluescript SK+ vector containing full-length NFAT5 using restriction enzymes BamHI and XhoI and ligated into pcDNA3.1 vector. The inhibitory construct for NFAT5 or NFAT1 was designed using an shRNA-expressing construct targeting exon 8 of murine NFAT5 (U6-N5-ex8) or NFAT1 (U6-N1 ex8) under the control of the murine U6 small nuclear (sn)RNA promoter as described previously. Subcloning of EGFP, U6-N5-ex8 or U6-N1-ex8 into a pLKO.1 vector and cotransfecting HEK293-T cells with pLKO.1 was performed to generate lentivirus encoding EGFP, U6-N5-ex8 or U6-N1-ex8. psPAX2 and pMD2.G plasmids were used for preparation of lentivirus (Addgene Inc. MIT, Cambridge, MA, USA).
**Lentivirus preparation**

DNA for transfection was prepared by mixing 1.0 µg psPAX2 and 0.1 µg pMD2.G with 1.5 µg pLKO.1 or psiLV plasmids in each flask. A mixture of 150 µl OptiMEM (Gibco) and 6 µl FUGENE (Roche) were then added to the DNA and this mixture was incubated for 15 min before addition to the packaging cells.

**Gene transfer in vivo**

Mice were anesthetized with 100 mg/kg ketamine-20 mg/kg xylazine (ip). In anesthetized mice, a 31G needle was inserted at the lower pole of the both kidneys parallel to the long axis and was carefully pushed toward the upper pole. As the needle was slowly removed, 50 µl filter-purified lentivirus (EGFP, U6-N5-ex8, or U6-N1-ex8 ~3x10^7 TUs) was injected into each kidney. Preliminary studies showed that lentiviral-mediated EGFP DNA and protein expression in kidney parenchyma were robust after 72 h.

**Transient transfection of mTAL cells**

Primary cultures of mTAL cells were cultured to 70–80% confluence as indicated, and cells were placed in 1 ml of serum-free OPTI-MEM medium containing different plasmid DNA constructs and 10 µl Lipofectamine 2000 (Invitrogen). Transfection efficiency was evaluated by flow cytometry as described previously.

**Model of renal IRI**

After mice were anesthetized, they were placed on a heated surgical pad to maintain a constant body temperature and the left renal pedicle was clamped for 30 min with atraumatic microvascular clips (FE 723 K, Aesculap, Tuttlingen, Germany) to induce ischemia, which was verified by the change of renal color. During the period of the left kidney clamping, and incision suturing, the core body temperature was maintained constant at 36°C by placing the mice on electric heating pads. In the sham group, clamps were not applied after incision. After removal of the clamp, the mice were sacrificed at 3 and 48h after reperfusion. Kidney tissue was divided to be either snap frozen for subsequent mRNA extraction or to be fixed in 4% neutral-buffered paraformaldehyde overnight for paraffin embedding.

**Isolation of total RNA and amplification of cDNA fragments**

Total RNA was isolated from medulla, mTAL tubules and primary cultures of mTAL cells by adding 1 ml TRIzol Reagent. After total RNA was treated with deoxyribonuclease I for 30 min, a 3 µg aliquot was used for cDNA synthesis using the Superscript Preamplification system (Life Technologies). cDNA fragments were sequenced or size fractionated on a 1% agarose gel and stained with ethidium bromide.

**Quantitative Real Time Reverse Transcription-PCR analysis**

A 0.5 µg aliquot of total RNA was converted to cDNA and placed in a 20 µl RT-PCR mixture using a FastStart DNA Master SYBR Green I kit (Roche) supplemented with 3 mM MgCl2 and Platinum Taq polymerase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was used to determine the accumulation of mRNA. The specific primer pairs for murine NFAT5, NFAT1, EGFP, NGAL and Kim-1 are provided in Table...
S1. Input cDNAs were normalized using the housekeeping gene, β-actin, and the efficiency of primer pair amplification determined using a standard curve generated. The 2(-delta delta CT) method was used to evaluate changes in mRNA accumulation for NFAT5, NFAT1, SMIT, NGAL and Kim-1.

**Western Blot Analysis**
Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Following blocking at for 1 h with 5% skim milk, membranes were probed at 4°C overnight with appropriate primary antibodies, followed by incubation with horseradish peroxidase (HP)-conjugated secondary antibodies (Amersham Pharmacia Biotech). Membranes were washed, and proteins detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**Immunohistochemistry Analysis**
Kidneys were perfusion fixed in situ with 4% paraformaldehyde, fixed in 4% neutral-buffered paraformaldehyde overnight, and embedded in paraffin wax. The sections were stained with hematoxylin and eosin and examined by light microscopy (Nikon Eclipse, TE2000-U). Alternatively, sections were incubated in a blocking solution consisting of PBS with 1% BSA for 1 h in a humidified chamber at room temperature then treated sequentially with either primary anti-EGFP polyclonal rabbit antibodies (1:500 dilution) and secondary antibody diluted 1:10,000 in PBS (donkey anti-rabbit-conjugated with Alexa Fluor 488; Invitrogen).

For the caspase-3 assay, tissue sections were permeabilized with 0.1% Triton X-100 for 30 min at RT, and incubated overnight at 22°C with a primary antiserum against caspase-3. The secondary antibody and corresponding peroxidase-anti-peroxidase (PAP) complex were applied for 30 min each at 22°C. The immunoperoxidase reaction was visualized after incubation of sections in 0.1% (wt/vol) diaminobenzidine and 0.03% hydrogen peroxide. Controls for the immunostaining procedure were prepared by omission of the first antibody or by replacing it with preimmune serum. Sections were visualized using a Nikon Microphot FXA microscope.

**TUNEL staining**
An ApopTag-fluorescein in situ DNA fragmentation detection kit (Chemicon, CA, USA) was used to visualize TUNEL-labeled fluorescence on paraffin slides. Briefly, tissue sections were incubated with proteinase K for 20 minutes and TUNEL labeling was performed using an in situ method. The relative number of apoptotic cells in different regions of the kidney was assessed per the same area for each sample by measuring the TUNEL- and propidium iodide (PI)- nuclear fluorescece intensity with laser-scanning cytometry (LSC; iCys; CompuCyte, Westwood, MA). Other details of the LSC-assisted analysis are presented elsewhere.
References Cited


Table S1: Oligonucleotide specific primers for qRT-PCR

<table>
<thead>
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<th>Primer</th>
<th>sequence</th>
<th>PCR size</th>
<th>Position</th>
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<td>F-NFAT5(m)</td>
<td>5'-AACATTGGACAGCCAAAAAGG-3</td>
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<tr>
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<tr>
<td>F-NGAL(m)</td>
<td>5'-GGCAGCTTTACGATGTACAG-3</td>
<td>221-240</td>
<td></td>
</tr>
<tr>
<td>R-NGAL(m)</td>
<td>5'-GCCACTTGCACATTGATGCT-3</td>
<td>420-401</td>
<td></td>
</tr>
<tr>
<td>F-KIM1(m)</td>
<td>5'-GGCTTCCTATGTTGCGATCTG-3</td>
<td>718-737</td>
<td></td>
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<tr>
<td>R-KIM1(m)</td>
<td>5'-GTTGCTTCAGCTCGGAAT-3</td>
<td>900-881</td>
<td></td>
</tr>
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