A Bioinformatics Approach Identifies Signal Transducer and Activator of Transcription-3 and Checkpoint Kinase 1 as Upstream Regulators of Kidney Injury Molecule-1 after Kidney Injury

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ABSTRACT

Kidney injury molecule-1 (KIM-1)/T cell Ig and mucin domain-containing protein-1 (TIM-1) is upregulated more than other proteins after AKI, and it is highly expressed in renal damage of various etiologies. In this capacity, KIM-1/TIM-1 acts as a phosphatidylserine receptor on the surface of injured proximal tubular epithelial cells, mediating phagocytosis of apoptotic cells, and it may also act as a costimulatory molecule for immune cells. Despite recognition of KIM-1 as an important therapeutic target for kidney disease, the regulators of KIM-1 transcription in the kidney remain unknown. Using a bioinformatics approach, we identified upstream regulators of KIM-1 after AKI. In response to tubular injury in rat and human kidneys or oxidant stress in human proximal tubular epithelial cells (HPTECs), KIM-1 expression increased significantly in a manner that corresponded temporally and regionally with increased phosphorylation of checkpoint kinase 1 (Chk1) and STAT3. Both ischemic and oxidant stress resulted in a dramatic increase in reactive oxygen species that phosphorylated and activated Chk1, which subsequently bound to STAT3, phosphorylating it at \$727. Furthermore, \$TAT3 bound to the KIM-1 promoter after ischemic and oxidant stress, and pharmacological or genetic induction of STAT3 in HPTECs increased KIM-1 mRNA and protein levels. Conversely, inhibition of STAT3 using siRNAs or dominant negative mutants reduced KIM-1 expression in a kidney cancer cell line (769-P) that expresses high basal levels of KIM-1. These observations highlight Chk1 and STAT3 as critical upstream regulators of KIM-1 expression after AKI and may suggest novel approaches for therapeutic intervention.

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Genome-wide expression analysis of the kidneys after kidney damage caused by ischemic¹ or toxic^{2,3} insults has revealed that *Kidney Injury Molecule-1* (*KIM-1*) mRNA levels are elevated more than any other gene. KIM-1 (also known as T cell Ig and mucin [TIM-1]) and hepatitis A virus cellular receptor 1 are type I cell membrane glycoproteins containing a unique six-cysteine Ig-like domain and a mucin-rich extracellular region that is conserved across zebrafish, rodents, dogs, primates, and humans.^{1,3} KIM-1 protein is localized at very high levels on the apical membrane of proximal tubular

epithelial cells in most injured regions of the kidney.^{1,4} After injury, the ectodomain of KIM-1 is shed

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in the urine and serves as a sensitive and early diagnostic indicator of proximal tubular injury in rodents and humans.^{3,5} Functionally, KIM-1 has been identified as a phosphatidylserine receptor on the proximal tubular epithelial cells that recognizes and mediates phagocytosis of necrotic and apoptotic cells as well as oxidized lipoproteins.⁶ In addition to various forms of AKI, KIM-1 is also highly expressed in chronic kidney injury,7 renal cell carcinoma,8 polycystic kidney disease9 and diabetic nephropathy.10 KIM-1/TIM-1 is also preferentially expressed by T helper 2 immune cells11,12 and regulates effector T cell function.13 TIM-1 has been found on immune cells, such as mast,¹⁴ B,¹⁵ and invariant natural killer T cells.¹⁶ As a result, KIM-1/TIM-1 has been identified as an important therapeutic target in immune diseases,17 allograft rejection,18 kidney disease,19 and kidney toxicity.20 A number of ligands for KIM-1/ TIM-1 and downstream signaling has been shown depending on the cell type and disease state.¹² Although significant progress continues to be made to understanding the potential significance of KIM-1 expression and its downstream signaling, relatively little is known about the mechanisms responsible for transcriptional regulation of KIM-1. Therefore, the objective of this study was to take a comprehensive computational and experimental approach in identifying the molecular regulators of KIM-1.

We applied chromatin immunoprecipitation enrichment analysis (ChEA)²¹ as well as kinase enrichment analysis (KEA)²² to previously generated gene expression profiles from the rat kidney after ischemia reperfusion injury (IRI)^{23,24} and identified signal transducer and activator of transcription-3 (STAT3) and checkpoint kinase 1 (Chk1) as potential regulators of KIM-1. Because STAT3 and Chk1 play a critical role in DNA damage signaling response, we hypothesized and experimentally validated the activation and interaction of Chk1, STAT3, and KIM-1 expression after kidney IRI in rats or oxidant stress in human proximal tubular epithelial cells.

RESULTS

A Bioinformatics Approach to Identify Transcription Factors and Kinases That Regulate KIM-1

We had previously conducted temporal gene expression profiling in the cortex and medulla of rat kidney (n=3/time point) after kidney IRI.^{23,24} In highly variable expressed genes across different time points after IRI (median absolute deviation>0.4; n=1571), we performed hierarchical clustering based on all time points in both datasets and then selected a set of probes (n=220) that showed upregulation of gene expression in early time points after IRI (6 and 24 hours) in both kidney cortex and medulla (Figure 1A). After removing the redundant genes from 220 probes, 174 probes were tested for the enrichment of known targets for 290 transcription factors using ChEA.²¹ Of the top 10 transcription factors (Table 1), only 6 transcription factors were profiled in our expression array, and of these transcription factors, only 3 transcription factors (V-myc avian myelocytomatosis viral oncogene analog [MYC], Kruffer like factor 4 [KLF4], and STAT3) were upregulated during IRI (Figure 1B). The potential targets of these transcription factors were also identified from ChEA (Table 1); in addition, because the set of transcription factors identified from a specific database was incomplete because of cell type- and condition-specific biases, we also applied algorithm for the reconstruction of accurate cellular networks (ARACNe)²⁵ to the entire expression profiling data to identify another 8 and 20 targets of MYC and STAT3, respectively (0 targets for KLF4) (Table 1). Because the hierarchical clustering on a whole expression profile can be biased by tissue origins, such as medulla or cortex, we also performed significance analysis of microarrays and confirmed STAT3 to be among the top 10 enriched transcription factor candidates (Supplemental Figure 1, Supplemental Table 1). Figure 1C shows the IRI-associated genes also represented as targets of three transcription factors (MYC, KLF4, and STAT3) from either ChEA or ARACNe analysis. Almost half of the selected genes are potentially regulated by more than one transcription factor (inner layer of the network) (Figure 1C). STAT3 and KLF4 both have MYC as their potential target. KIM-1 was identified as a potential transcriptional target of STAT3 by ARACNe analysis. To predict the potential binding sites of the candidate transcription factors, we used evolutionary conserved region database.²⁶ Among 209 sequencebased predictions, we found 5 potential binding sites for V-myb avian myeloblastosis viral oncogene homolog, MYC, STAT3, E2F transcription factor 4, and Forkhead box M1 transcription factors (Figure 1D).

To identify the upstream kinases mediating KIM-1 induction, we first used Genes2Networks,27 which represents a collection of potential gene-gene relationships obtained from diverse cellular and experimental conditions. To fully exploit the database, we included all eight transcription factors identified by ChEA analysis to generate a global interactive network and minimize the potential loss of information (Figure 1E). We then performed KEA²² to curate kinases with known targets that are enriched for the selected transcription factors as well as their interacting genes reported by Genes2Networks analysis (Figure 1F). The top three scoring kinases were mitogen-activated protein kinase 1 (MAPK1; P<0.001), cyclindependent kinase 4 (CDK4; P<0.001), and Chk1 (P<0.001). Because relatively little is known about the role of Chk1 after kidney IRI, we considered the hypothesis that IRI results in the generation of reactive oxygen species (ROS)²⁸ that triggers DNA damage, causing activation of Chk1. We also hypothesized that activated Chk1 interacts with STAT3 to phosphorylate it and that phosphorylated STAT3 binds on the KIM-1 promoter for its transcription (Figure 1G).

ROS-Mediated DNA Damage Activates Chk1 after Kidney IRI

After 30 minutes bilateral kidney IRI, there was \sim 10-fold increase in ROS as early as 3 hours that peaked at 12–24 hours in the rat kidney cortex and medulla (Figure 2A). Protein expression as well as phosphorylation of Chk1 also significantly



Figure 1. Bioinformatics approach identifies STAT3 and Chk1 as molecular regulators of KIM-1 expression after IRI. (A) Left heat map panel shows genes with highly variable expression (n=1571) across various time points after 20 minutes of bilateral IRI (red and blue indicate relative up- and downregulation, respectively). Hierarchical clustering was performed by using 1–Pearson correlation as distance with average linkage. According to the hierarchical clustering, we selected 220 probes showing substantial upregulation in early time points (6 and 24 hours after IRI) both in renal cortex and medulla (right heat map panel). (B) Six transcription factors with expression that is available in our dataset are shown across different time points after IRI (solid and dashed lines are cortex and medulla, respectively). (C) The IRI genes associated with each of three transcription factors (MYC, KLF4, and STAT3) that showed upregulation after IRI are shown as network presentation. The IRI genes with single connection with each of the three transcription factors are shown in outer layer. KIM-1 (yellow) is identified as a target of STAT3 using ARACNe. (D) Potential transcription factor binding sites on KIM-1 promoter sequence. (E) Five transcription factors (E2F transcription factor 4 [E2F4], STAT3, MYC, V-myb avian myeloblastosis viral oncogene homolog [MYB], and KLF4; red nodes) are shown with their interacting genes as visualized by Genes2Networks. The transcription factors without interacting gene partners are ignored in this analysis. (F) Top 10 kinases are shown for their significance (-loq₁₀P) of enrichment (*i.e.*, the overlap between the known targets of the kinase and genes identified in Genes2Networks). (G) Based on literature and our bioinformatics predictions, the schematic diagram represents our hypothesis that IRI generates ROS, thereby initiating DNA damage signaling response in the kidney. Chk1 is activated, and it binds and phosphorylates STAT3, which in turn, translocates to the nucleus and binds to KIM-1 promoter, resulting in its transcription.

increased at 3 hours and remained elevated until 24 hours in the cortex, whereas it peaked at 48 hours in the medulla, corresponding to the presence of other DNA damage protein kinases phospho-ataxia telangiectasia and Rad3 related (pATR) and phospho-histone family member X (pH2A.X) (Figure 2B). Immunostaining for pH2A.X-positive foci peaked at 12 hours and was persistent until 48 hours (Figure 2C). Persistent ROS-mediated DNA damage resulted in elevated kidney dysfunction, which was measured by serum creatinine and BUN (Supplemental Figure 2A), proximal tubular necrosis (Supplemental Figure 2B), and apoptosis (Figure 2D), all of which peaked at 12–24 hours. Because proximal tubular epithelial cells are the primary targets of IRI, as a model, we used human proximal tubular cells (HPTECs) treated with 1 mM hydroxyurea (HU), which resulted in 30% cell death (Supplemental Figure 3). This treatment showed an approximately threefold induction of ROS (Figure 2E) and significant increase in pATR, phospho-checkpoint kinase 1 (pChk1), and pH2A.X (Figure 2F), culminating in apoptosis (Figure 2G). Furthermore, YCG063, a specific inhibitor of ROS (Supplemental Figure 4), was able to reduce the HU-mediated phosphorylation of Chk1 (Figure 2H). These results suggest that proximal tubular epithelial cell injury results in ROS generation that activates DNA damage signaling, such as Chk1, leading to cell cycle arrest and apoptosis (Figure 2I).

Chk1 Binds to STAT3 and Phosphorylates It after Oxidant Stress in HPTECs

To determine if Chk1 regulates activation of STAT3 and KIM-1 expression, we pretreated the HPTECs with a selective inhibitor of Chk1, SB218078, followed by 1 mM HU treatment.

SB218078 inhibited the HU-mediated phosphorylation of Chk1 and decreased the phosphorylation of STAT3 at S727 as well as Y705 (Figure 3A). KIM-1 expression was also decreased after SB218078 treatment, suggesting a positive regulation between pChk1, phospho-signal transducer and activator of transcription (pSTAT3) S727, and KIM-1. Although phosphorylation of Janus-activated kinase-2 (pJAK2) increased after HU treatment, SB218078 treatment did not alter pJAK2 expression, suggesting the specificity of SB218078 for Chk1 (Figure 3A). Similarly, Chk1 small interfering RNA (siRNA) also decreased induction of pChK1, KIM-1, and phosphorylation of STAT3 at S727 and Y705 after HU treatment (Figure 3B). Furthermore, to rule out the possibility that

Table 1. Top 10 significant enrichments with IRI targets in ChEA analysis

TF	Reference (Pubmed)	Target	P Value	Genes
MYC	18358816	65	<0.001	ADA, ANGPTL4, ARHGAP11A, ARL2, ARPC3, ATF3, AURKB, BANF1, CD68, CDC20, CDC2A, CDCA1, CKS1B, CKS2, CTPS, CTSD, DDX39, DUSP6, EIF4EBP1, EMP3, GMNN, H2AFX, HMOX1, IER2, IER5L, JUNB, KIF20A, KIF22, KRTCAP2, LDHA, LSM4, MAP2K3, MED19, MRPL23, MRPS18A, NME1, NPM3, NRM, NUBP1, NUBP2, PPP1R14B, PRC1, PSMB10, PSMC3, RAB3D, RHOA, RIPK3, RPL10A, RPL27A, RPL37, RPS9, RRM2, SIVA, SNRPA, SRM, TAF15, TGIF, TMED5, TMEM97, TRAPPC1, TUBA4, TUBB5, UBE2C, UMPS, VARS2
MYC	19079543	32	<0.001	ALKBH6, ANGPTL4, ARHGAP11A, BANF1, BTG2, CD68, CDCA1, CKS1B, CKS2, DDX39, EIF4EBP1, HMGB2, IER2, KIF20A, LDHA, NME1, NUBP2, PLP2, PPAP2C, PRKCDBP, PSMB6, RHOA, RPL27A, RPL37, RPS9, SNRPA, SRM, TGIF, TMEM97, TSN, TUBB5, UMPS
KLF4	18358816	33	<0.001	ADA, ATF3, BTG2, CKS1B, CLIC1, CXCL16, DUSP6, ECT2, EGR1, FOSL1, GMNN, H2AFX, IER2, JAK3, JUNB, KIF20A, KRT8, KRTCAP2, LSM4, MAP3K1, MYC, NQO1, NRM, PPP1R14B, PRKCDBP, RAB3D, RIPK3, TGIF, TMEM97, TUBB2A, TUBB5, UBE2C, WFDC2
MYC	18555785	26	<0.001	ANGPTL4, BANF1, CKS1B, CTPS, DDX39, EIF4EBP1, H2AFX, HMOX1, KIF20A, LSM4, MRPL23, MRTO4, NME1, NPM3, NUBP1, NUBP2, PRC1, RPL10A, RPL27A, RPL37, RPS9, RRM2, SNRPA, TAF15, TMEM97, UMPS
E2F4	17652178	23	<0.001	AURKB, CCNB2, CDCA1, DRG1, FOS, GMNN, H2AFX, HMGB2, JUNB, KIF20A, LSM3, LSM4, MYC, PBK, PRIM1, PSMC3, RFC2, RRM2, SNRPA, SSR2, TGIF, TMED5, UMPS
EKLF	21900194	25	<0.001	BTG2, CDC20, CLIC1, DDX39, EIF4EBP1, EMP3, HMOX1, KRTCAP2, LDHA, MYC, NQO1, NUBP2, PSME2, RFC2, RPL10A, RPP21, RPS9, SH3BGRL3, SNRPA, SRM, SSR2, TUBB5, UBE2C, UMPS, ZNRD1
TRP63	18441228	11	<0.001	AURKB, CKS1B, COPEB, CYP3A13, FOS, FOSL1, HMOX1, JUN, KIF23, PHLDA1, RRM2
MYB	21317192	20	<0.001	DUSP6, EMP3, FXYD5, H2AFX, JUNB, MAP2K3, MYC, NFIL3, PHLDA1, PLP2, RAB3D, RHOG, RHOJ, RRM2, SERTAD1, SLPI, SRM, SSR2, TMEM97, UMPS
CHD1	19587682	17	<0.001	ALKBH6, ARHGAP11A, DRG1, DUSP6, EMP3, IER2, KIF20A, LSM4, N6AMT2, RPL27A, RPL37, RPS9, RRM2, SNRPA, TMEM160, TRAPPC1, TUBB5
STAT3	1855785	14	<0.001	ADA, FOS, HMGB2, IER2, JAK3, JUNB, LSM4, MAP3K1, MYC, NME1, SPC24, SPSB1, TUBB5, WFDC2
MYC	ARACNe	8	< 0.001	BTG2, TNFRSF12A, COPEB, LOC313722, ATF3, IER2, JUNB, GADD45G
STAT3	ARACNe	20	<0.001	LOC497665 (KIM-1), BTG2, TNFRSF12A, PLP2, PVR, CD14, RASD1, COPEB, CTPS, LOC313722, CSRP3, TAX1BP3, TGIF, IER2, ARPC3, LDHA, LOC500689, JUNB,

For 10 significant enrichments identified from ChEA, the corresponding transcription factors (TFs), reference PubMed identification as available from the ChEA database, number of IRI targets (174 symbols) in curated targets, P value from Fisher exact test, and IRI target genes are shown. Top 10 enrichments correspond to eight TFs, because MYC is presented three times (each enrichment corresponds to different target sets from independent experiments). Expression-based IRI target sets identified using ARACNe analysis (8 and 20 IRI targets of MYC and STAT3, respectively, and 0 IRI targets for KLF4; all three TFs showed concordant upregulation themselves during IRI process) are listed below. E2F4, E2F transcription factor 4; MYB, V-myb avian myeloblastosis viral oncogene homolog.



Figure 2. IRI in the kidney activates Chk1 as a result of DNA damage. After 30 minutes IRI in rats, kidney tissue lysates were prepared, and (A) ROS estimation was plotted as DCFDA converted to DCF. (B) Immunoblotting and respective quantitation analysis of pATR, pChk1 and pH2A.X. α -Tubulin served as loading control. (C) pH2A.X immunostaining (green) in kidney sections (left panels). Scale bar: 10 μ m. The number of pH2A.X foci per nuclei was plotted (right panels). (D) DNA damage by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay (green) in kidney sections (left panel). Scale bar: 10 μ m. Percentage of TUNEL-positive nuclei were plotted (right panel). HPTECs were treated with HU and (E) incubated with DCFDA for 1 hour. Cells were imaged immediately. Scale bar: 10 μ m. Mean fluorescence intensity (MFI) was plotted for Ctrl and HU-treated cells. (F) Immunoblotting was performed for pATR, pH2A.X, pChk1, and Chk1. GAPDH served as loading control. (G) DNA damage determined by TUNEL assay (green). Scale bar: 10 μ m. Percentage of TUNEL-positive nuclei was performed in HPTECs treated with ROS inhibitor YCG063 alone or combined with HU. (I) Schematic diagram summarizing the results in this figure. ROS generation caused by IRI or oxidant stress (HU treatment) in the proximal tubular epithelial cells of kidney mediates DNA damage signaling response, causing phosphorylation of ATR and Chk1. *Significance compared with treatment (*P*<0.05).

pJAK2 did not mediate STAT3 phosphorylation, we pretreated HPTECs with a specific JAK inhibitor, AG490, followed by 1 mM HU treatment. The results suggested that inhibition of HU-activated pJAK2 by AG490 decreased STAT3 Y705 phosphorylation (Figure 3C). KIM-1 expression under similar conditions was not inhibited after AG490-mediated inhibition of activated pJAK2 (Figure 3C). We next asked whether Chk1 interacts with STAT3 in HPTECs after oxidant stress. We immunoprecipitated Chk1 with its specific antibody and immunoblotted for STAT3, pSTAT3 S727, or pSTAT3 Y705 in HPTECs after 1 mM HU treatment and Chk1 inhibitor pretreatment. Results suggest specific binding of Chk1 with STAT3 and more binding to the S727-phosphorylated form of STAT3 than the Y705-phosphorylated form (Figure 3D). We also confirmed the interaction between pSTAT3 S727 and pChk1 by immunostaining (Figure 3E) and confocal imaging (Supplemental Figure 5) in HPTECs and observed significant increases in colocalization after HU treatment that decreased after addition of SB218078. This information provides strong evidence for previously unrecognized interaction between Chk1 and pSTAT3 and their role as DNA damage signaling after kidney injury (Figure 3F).

STAT3 Activation and KIM-1 Expression Positively Correlate in Rat and Human Kidneys after Acute Tubular Injury

We then wanted to evaluate the temporal expression and correlation between STAT3 and KIM-1 after AKI. After 30 minutes bilateral IRI in rats, KIM-1 mRNA (Figure 4A) and protein expression (Figure 4B) in the kidney cortex as well as medulla significantly increased as early as 6 hours and peaked at 24 hours (maximum injury) before declining at 48 hours (Figure 4, A and B). STAT3 S727 phosphorylation significantly increased at 3 hours in the kidney cortex and medulla after IRI and showed robust expression throughout the time course until 48 hours (Figure 4B). In contrast, STAT3 Y705 showed more robust phosphorylation in the medulla compared with cortex. JAK2, one of the upstream kinases of STAT3, was not phosphorylated in early time points, confirming the involvement of another kinase (Chk1) in phosphorylation of STAT3, which showed increased



Figure 3. Chk1 binds to STAT3 and activates it after kidney injury. HPTECs were pretreated with (A) 1 μ M Chk1 inhibitor (SB218078) and/or HU, and its effect on expression of pChk1, Chk1, pJAK2, JAK2, pSTAT3 Y705, pSTAT3 S727, STAT3, and KIM-1 was assessed using immunoblotting and quantitative analysis. (B) HU and/or Ctrl or Chk1 siRNA and its effect on expression of pChk1, Chk1, pSTAT3 Y705, pSTAT3 S727, STAT3, and KIM-1 were assessed using immunoblotting and quantitative analysis. (C) HPTECs were treated with HU and/or JAK2 inhibitor (AG490), and its effect on expression of pJAK2, JAK2, pSTAT3 Y705, pSTAT3 S727, STAT3, and KIM-1 was assessed using immunoblotting followed by quantitative analysis. GAPDH served as loading control. (D) HPTECs were treated with HU alone or combined with SB218078, and immunoprecipitation by Chk1 antibody was performed followed by immunoblotting for pSTAT3 Y705, pSTAT3 S727, or STAT3. GAPDH and IgG light chain served as loading controls for input and IP, respectively. (E) Immunofluorescence was performed to evaluate expression of pSTAT3 S727 and pChk1 in HPTECs. Images were obtained at 63× objective using an immunofluorescent microscope. Scale bar: 10 μ m. (F) Schematic diagram advancing the hypothesis and summarizing the results in this figure that shows binding of Chk1 and STAT3, resulting in its phosphorylation. *Significance compared with reatment (*P*<0.05).

phosphorylation as early as 3 hours (Figure 2B). Furthermore, we investigated the correlation between KIM-1 and STAT3 phosphorylation in human kidney sections obtained from patients with or without acute tubular injury (ATI) (Figure 4C). The staining pattern for pSTAT3 S727 and Y705 was observed in the epithelium along the entire tubular portion of the nephron from proximal to the distal tubule and not in the collecting ducts. Representative images for pSTAT3 Y727 and S705 staining from the medulla of ATI patients are shown in Supplemental Figure 6, A and B, respectively. There was 74% pSTAT3 Y705, 81% pSTAT3 S727-positive nuclei, and 35% KIM-1-positive tubules in ATI patients compared with 3.3% pSTAT3 Y705, 0.8% pSTAT3 S727-positive nuclei, and 0.6% KIM-1-positive tubules in non-ATI patients. All the KIM-1-positive tubules were positive for pSTAT3 staining (Figure 4C), suggesting a strong correlation between the STAT3 phosphorylation and KIM-1 expression after kidney tubular injury.

STAT3 Binds to the KIM-1 Promoter and Regulates Its Expression

To test our hypothesis that the transcription factor STAT3 binds to the KIM-1 promoter, we evaluated promoter binding activity for STAT3 on the KIM-1 promoter in rat kidney after IRI by chromatin immunoprecipitation (ChIP) assay. The results suggested an increased DNA binding on the KIM-1 promoter at 12 hours in cortex and 6 hours in medulla, which was sustained until 24 hours, suggesting STAT3 DNA binding on the KIM-1 promoter after IRI (Figure 5A). We next examined the DNA binding activity of STAT3 on the KIM-1 promoter by luciferase assay using not only HPTECs but also, 769-P cells, a human renal cell carcinoma cell line, expressing very high levels of pSTAT3 and KIM-1 (Figure 5B). The HPTECs showed significantly higher KIM-1 luciferase activity after the addition of IL-6 and dexamethasone (STAT3 inducers) and after STAT3 transfection or HU treatment (Figure 5C), whereas KIM-1



Figure 4. Expression of KIM-1 and STAT3 phosphorylation is correlated in rat kidneys after IRI and patients with ATI. After 30 minutes IRI in rat cortex and medulla, (A) RT-PCR was performed for KIM-1 mRNA, and the results were normalized to GAPDH. *Significance compared with sham (P<0.05). (B) Immunoblotting and respective quantitation analysis for expression of pSTAT3 (Y705, S727), STAT3, pJAK2, JAK2, and KIM-1 were performed. β -Actin served as loading control. (C) Human kidney biopsies with or without ATI were costained for pSTAT3 (Y705, S727) and KIM-1 (left panels). Scale bar: 10 μ m. Area and tabular chart of percentage pSTAT3 nuclei and percent KIM-1–positive tubule (right panel).

mutant luciferase showed significant decrease in luciferase activity but not complete inhibition (Figure 5C). As expected, 769-P cells showed significantly high KIM-1 luciferase activity, which was significantly reduced after transfection with STAT3 siRNA (Figure 5C).

To evaluate the regulatory role of STAT3 in transcription and translation of KIM-1, we transfected HPTECs with STAT3 mutants (STAT3_Y705F or STAT3_S727A) and treated them with IL-6 and dexamethasone. We observed decreased KIM-1 mRNA (Figure 5D) and protein (Figure 5E, Supplemental Figures 7A and 8A) in STAT3_Y705F as well as STAT3_S727A-transfected HPTECs. Furthermore, there was significant downregulation of KIM-1 mRNA and protein expression in 769-P cells transfected with STAT3 siRNA (Figure 5, F and G, Supplemental Figures 7B and 8B). Transfecting 769-P cells with STAT3 mutants (Y705F or S727A) resulted in decreased KIM-1 expression (Figure 5G, Supplemental Figure 7B). To confirm the specific role of S727 phosphorylation on Chk1-mediated KIM-1 expression, we transfected HPTECs with STAT3_S727A plasmid, and treatment of these cells with HU resulted in significantly decrease KIM-1 expression (Figure 5H). Taken together, these results confirm STAT3 occupancy on the KIM-1 promoter and the regulation of KIM-1 transcription and translation by activation of STAT3 (Figure 5I).

DISCUSSION

CKD and AKI are major public health problems that affect over 30 million adults in the United States.²⁹ The incidence of AKI is increasing, with over 1 million cases each year in the United States, and mortality rates remain high.^{30–33} Given that *KIM-1* is the most upregulated gene in proximal tubular epithelium after AKI or CKD, understanding the mechanisms regulating KIM-1 might offer novel therapeutic control points as mitigation strategies for AKI or CKD. Here, we report four findings. (1) Chk1 is significantly increased temporally in response to ROS generation in the rat kidney cortex and medulla after IRI as well as the HPTECs after oxidant stress. (2) Activated Chk1 binds to STAT3 in the HPTECs after oxidant stress, phosphorylating it at S727, and inhibition of Chk1 in HPTECs reduces STAT3 phosphorylation as well as KIM-1 expression. (3) Increased STAT3 phosphorylation (Y705 and S727)



Figure 5. STAT3 binds on the KIM-1 promoter and regulates its expression. (A) ChIP assay was performed in kidney tissue for STAT3 binding on the *KIM-1* promoter. (B) Immunoblotting for KIM-1, pSTAT3, STAT3, and α-tubulin (loading control) was performed in HPTECs and renal epithelial carcinoma cells (769-P) to evaluate endogenous levels of these proteins. (C) KIM-1 luciferase assay was performed with wild-type or mutant KIM-1 luciferase in HPTECs after transfection with STAT3 plasmid and treatment with IL-6 and dexamethasone or HU. KIM-1 wild-type and mutant luciferase activity was also measured in 769-P cells transfected with STAT3 siRNA. (D) RT-PCR analysis for *KIM-1* mRNA was performed in HPTECs treated with IL-6 and dexamethasone and transfected with empty vector (EV) versus STAT3 plasmid or transfected with STAT3_S727A and/or IL-6 and dexamethasone treatment; 769-P cells served as positive control. (E) Immunoblotting and respective quantitation analysis for expression of KIM-1, pSTAT3 (Y705 and S727), and STAT3 were performed in HPTECs treated with IL-6 and dexamethasone. In addition, expression of these proteins was also evaluated in HPTECs transfected with STAT3_S727A alone or combined with IL-6 and dexamethasone. *α*-Tubulin served as loading control. (F and G) 769-P cells were transfected with Ctrl siRNA, STAT3 siRNA, EV, or STAT3 mutants (Y705F or S727A), and (F) RT-PCR analysis for *KIM-1* mRNA as well as (G) immunoblotting and respective quantitation analysis for KIM-1, pSTAT3 (Y705 and S727A), and S727), and STAT3 were performed. *α*-Tubulin served as loading control. (H) HPTECs transfected with EV or STAT3_S727A with HU

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correlates significantly with KIM-1 expression after IRI in rats as well as kidney biopsies of ATI patients. (4) STAT3 binds to the KIM-1 promoter and regulates its transcription in rat kidneys after IRI, HPTECs after oxidant stress, and renal cell carcinoma cells, which have very high basal levels of KIM-1. These results not only offer novel mechanistic insights about regulation of KIM-1, but also allude to newer therapeutic strategies in modulating KIM-1 expression for AKI and chronic kidney injury, kidney cancer, polycystic kidney disease, and diabetic nephropathy as well as immune disorders and atopic diseases, where KIM-1/TIM-1 is highly expressed and thought to play a critical disease modulatory role in determining the final outcome.

A similar computational approach was recently taken to identify protein kinases that regulate gene expression changes in the kidneys of HIV transgenic 26 mouse line (Tg26 mice), which have both tubulointerstitial fibrosis and glomerulosclerosis.²⁸ This study identified homeodomain-interacting protein kinase 2 as an important regulator of kidney fibrosis but also found Chk1 as one of the top five kinases regulating kidney fibrosis. Using this approach, we found MAPK1 (extracellular regulated kinase 2), CDK4, and Chk1 as the top three protein kinases mediating protein phosphorylation after kidney IRI (Figure 1). The major kinase investigated here was Chk1, because considerable evidence exists about the role of MAPK1³⁴ and CDK4³⁵ in AKI, whereas relatively little is known about the role of Chk1 in kidney damage. Consistent with the role of checkpoint kinases in modulating DNA damage response,^{36,37} we found that ROS generated as a result of ischemic or oxidant stress in kidney tubular epithelium activated DNA damaging signaling proteins, such as pATR, pH2A.X, and pChk1, that resulted in apoptosis. We next investigated if Chk1 plays a functional role as an upstream kinase phosphorylating STAT3, because STAT3 was identified by ChEA and ARACNe analyses as the potential transcription factor regulating 220 highly upregulated genes after IRI. Inhibition of Chk1 phosphorylation after oxidant stress in HPTECs led to inhibition of phosphorylation of STAT3, suggesting the importance of Chk1 activation in the phosphorylation of STAT3 (Figure 3A). Furthermore, to confirm that only STAT3 Y705 phosphorylation is regulated by JAK2 in our cellular injury model, we inhibited JAK2 phosphorylation by physiologic inhibitor and found inhibition of only pSTAT3 Y705. The colocalization of pSTAT3 S727 with pChk1 in HPTECs after oxidant stress (Figure 3) along with the immunoprecipitation results showing more binding of S727 phosphorylated STAT3 than Y705 with Chk1 (Figure 3D) provide new evidence supporting Chk1-STAT3 interplay under DNA damage conditions in kidney tubular epithelial cells.

Although STAT3 has been shown to play an important role in kidney disease,³⁸ with inhibition of STAT3 being protective against kidney IRI³⁹ and kidney fibrosis,^{40,41} the significance of modulating Chk1 alone or the Chk1–STAT3 pathway after kidney damage remains to be investigated. Chk1 is a serine/ threonine kinase, and it is primarily responsible for initiating cell cycle arrest, allowing time for DNA repair and cell survival.⁴² In ischemic, toxic, and obstructive models of AKI, a causal association between epithelial cell cycle G2/M arrest and a fibrotic outcome was recently shown.⁴³ It would be interesting to investigate if modulating Chk1 alone or the Chk1–STAT3 pathway would help in preventing fibrosis by interrupting the progression of AKI to CKD.

KIM-1 was identified as a potential target of STAT3 by ChEA and ARACNe analyses. We observed a positive correlation between STAT3 activation and increased KIM-1 expression after ATI and in renal cell carcinoma. Only 50% of the pSTAT3positive cells exhibit KIM-1 staining, which may be because STAT3 (being an acute phase response protein⁴⁴) is expressed by injured, dedifferentiating, proliferating, and dying cells, whereas only dedifferentiating and surviving cells express KIM-1.6 Using in vivo and in vitro promoter binding assays, we were able to show that STAT3 binds to the KIM-1 promoter and regulates its transcription. KIM-1 mRNA and protein levels were modulated after induction of STAT3 in HPTECs and inhibition of STAT3 in renal cell carcinoma cells (769-P) using physiologic as well as genetic modification approaches. Given that STAT3 has only one predicted binding site on the KIM-1 promoter, which was mutated in the KIM-1 mutant luciferase construct, and the fact that we did not observe a complete inhibition of KIM-1 in the mutant (Figure 5C), we hypothesize that there could be more than one transcription factor regulating KIM-1 expression, which is consistent with the bioinformatics predictions (Figure 1D). Our results support the hypothesis that STAT3 and/or Chk1 (which binds to STAT3) could be new therapeutic control points for regulating KIM-1 expression. Anti-TIM-1/KIM-1 antibodies have been used as therapeutic candidates, which showed partial protection from cisplatin²⁰ or IRI-induced AKI⁴⁵ as well as crescentic GN,¹⁹ but this approach has been criticized for not having functionally characterized antibodies in terms of the specificity and selectivity to blocking KIM-1 function.⁴⁶ Because KIM-1/ TIM-1 has been recognized to play an important role in kidney disease as well as immunity and infection, it would be worthwhile to investigate whether modulating the Chk1-STAT3 axis to regulate the expression of KIM-1/TIM-1 has any effect in determining the outcome or slowing the progression of these diseases.

In summary, this study identifies Chk1 as an upstream kinase that interacts with STAT3 to initiate the DNA damage

treatment were immunoblotted and quantitated for KIM-1, pSTAT3 S727, and STAT3. α -Tubulin served as loading control. (I) Schematic summary of STAT3 DNA binding on the KIM-1 promoter. *Significantly different than respective controls (P<0.05). ^{\$}Significance compared with the same treatment.

signaling response in the kidney tubular epithelium after ischemic or oxidant stress. We also provide evidence for STAT3 binding to the KIM-1 promoter to regulate its transcription. These findings not only advance our molecular understanding of the process of kidney injury, but also provide novel insights in identifying targeted therapies for diseases where KIM-1/ TIM-1 has been shown to play a key role.

CONCISE METHODS

Animals

Male Wistar rats (280–320 g) were purchased from Harlan Laboratories (Indianapolis, IN). The animals were maintained in the central animal facility over wood chips free of any known chemical contaminants under conditions of $21\pm1^{\circ}$ C and 50%–80% relative humidity at all times in an alternating 12-hour light-to-dark cycle. Animals were fed with commercial rodent chow (Teklad Rodent Diet 7012), given water ad libitum, and acclimated for 1 week before use. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and they were approved by the Harvard Medical School Animal Care and Use Committees.

IRI in the Kidney

Male Wistar rats underwent 30 minutes of bilateral IRI as described before,^{23,24} whereas the sham-operated rats underwent anesthesia and a laparotomy only, and they were euthanized after 24 hours. Rats were euthanized by an overdose of phenobarbital (180 mg/kg) at 3, 6, 12, 24, or 48 hours after reperfusion (n=5/time point). Sagittal sections of kidney tissues were placed in 10% neutral buffered formalin and embedded into paraffin. For histologic evaluation, embedded tissues were cut into 4- to 6- μ m sections and stained with hematoxylin and eosin. Images were captured on a Carl Zeiss AxioImager.M2 using AxioVision SE64 software by Plan Apochromat 20×/0.8 objective. Serum creatinine and BUN were measured using a VetScan VS2 (Abaxis, Union City, CA).

Kidney Biopsy from Patients with and without ATI

Ten human kidney biopsy sections were obtained through Brigham and Women's Hospital's pathology service core, and they were classified as patients without evidence of ATI (n=5) and patients with evidence of ATI (n=5). The Institutional Review Board approved the protocol for extracting paraffin-embedded, formalin-fixed sections from patients with and without ATI.

Cells

HPTECs were purchased from Biopredic (Paris, France) and cultured in DMEM/F12 supplemented with hydrocortisone, EGF, insulin, transferrin, and sodium selenite on collagen-coated tissue culture plates. Renal epithelial carcinoma cell line 769-P was purchased from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS. Cells were maintained at 37°C in a humidified 5% CO₂ incubator.

Chemicals

DMEM, DMEM/F12 (1:1), and FBS were purchased from Life Technology (Invitrogen Corporation). Insulin transferrin and sodium selenium, collagen, and EGF were purchased from Invitrogen Corporation. SB218078, AG490, and YCG063 were purchased from Calbiochem (Philadelphia, PA). Dexamethasone and hydroxyurea was purchased from Sigma-Aldrich (St. Louis, MO). IL-6 was purchased from Preprotech (Rocky Hill, NJ), and Dharmafect1, DharmafectDuo, Control (Ctrl) siRNA, STAT3 siRNA, and Chk1 siRNA were purchased from Dharmacon, Inc. (Lafayette, CO). pGL3 Basic Luciferase plasmid and the Dual-Luciferase Reporter (DLR assay kit) were purchased from Promega (Madison, WI).

Plasmid Construction

A 900-bp fragment spanning 16-924 of the human KIM-1 promoter was PCR-amplified from genomic DNA of 769-P cells and cloned into the firefly luciferase-based pGL3-Basic vector (Promega) using the HD Fusion Kit from Clontech (Mountain View, CA) and primers as shown in Supplemental Table 2. Site-directed mutation at the STAT3 binding site was generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA) and primers as shown in Supplemental Table 2. These primers generate mutation of TT to the AA of STAT3 binding site, leading to the mutant KIM-1 promoter. The constructed plasmids were sequenced for the presence and accuracy of the cloned promoter sequence and mutation. Plasmid Renilla luciferase (pRL)-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Luciferase was a gift from Dr. Brock Matthias (Center of Experimental Rheumatology, University Hospital Zurich, Zurich, Switzerland). STAT3 wild-type cDNA cloned into the pRC/cytomegalovirus (CMV) plasmid pRC/CMV_STAT3;STAT3_Y705F construct contains tyrosine-substituted residues at Y705, and STAT3_S727A construct contains serine-substituted residues at S727.

Transfections

Semiconfluent (70%–80%) HPTECs were transfected with pRC/ CMV, pRC/CMV_STAT3, pRC/CMV_Y705F, or pRC/CMV_S727A plasmid using DharmafectDuo transfection reagent (as per the manufacturer's instruction), washed 6 hours post-transfection, and further incubated for 48 hours. Semiconfluent (70%–80%) 769-P cells were transfected with Ctrl or STAT3 siRNA using Dharmafect1 transfection reagent (as per the manufacturer's instruction), washed 6 hours post-transfection, and further incubated for 72 hours. HPTECs were transfected with Ctrl or Chk1 siRNA using Lipofetamine 2000 reagent (Invitrogen Corporation). Cells were washed 6 hours posttransfection and further incubated for 72 hours. Cells were then harvested and processed for RNA isolation followed by RT-PCR or immunoblotting.

Measurement of ROS Generation

ROS generation in kidney (cortex and medulla) was assayed using 2',7'-dichlorodihydrofluorescein diacetate, a nonpolar compound that, after conversion to a polar derivative by intracellular esterases, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein.^{47,48} Briefly, freshly prepared protein lysates were diluted 1:20 with ice-cold Locke's buffer (pH 7.4; 154 mM

NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 5.6 mM glucose, and 5 mM Hepes) to obtain a concentration of 5 μ g tissue/ μ l. Kinetic enzymatic reaction was carried out by incubating the lysate with 10 μ M 2',7'dichlorofluorescein diacetate (DCFDA) at room temperature, and 2',7'-dichlorofluorescein (DCF) formation was measured by a spectrofluorometer (Flex Station; Molecular Devices, Sunnyvale, CA) every 5 minutes at an excitation of 484 nm and an emission at 530 nm.^{47,48} Background subtractions were done from the DCFDA to DCF conversion in the absence of lysate. ROS generation in HPTECs was measured by fluorescence staining of DCFDA converted to fluorescent DCF as per the manufacturer's protocol.⁴⁹ In brief, after HU treatment with or without YCG063 (ROS inhibitor), cells were incubated with 10 μ M DCFDA for 1 hour. Cells were then imaged immediately on an Carl Zeiss AxioImager.M2 using AxioVision SE64 software by $63 \times /1.4$ oil differential interference contrast (DIC) objective.

Immunoblotting and Immunoprecipitation

Kidney tissues were homogenized in RIPA buffer (50 mM Tris·HCl [pH 7.4], 150 mM NaCl, 1% NP40, 1 mM PMSF, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, and 1× protease inhibitor cocktail [Roche Applied Science, Indianapolis, IN]), and equal protein (30 μ g) was resolved by PAGE. Proteins were transferred onto nitrocellulose membrane, and immunoblotting was performed with rabbit polyclonal anti-STAT3, pSTAT3 Y705, pSTAT3 S727, pJAK2 (Y1007/ 1008), JAK2, pATR (S428), pH2A.X (S139), anti-β-actin, mouse monoclonal (Cell Signaling Technology, Danvers, MA), rabbit polyclonal pChk1 (S317), goat polyclonal Chk1, KIM-1 (R&D Systems, Minneapolis, MN), anti– α -tubulin, and GAPDH (Sigma-Aldrich) antibodies. Horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, or goat were purchased from Jackson ImmunoResearch (West Grove, PA). Immunoprecipitation (IP) was performed as previously described.⁵⁰ Briefly, tissues were lysed in IP buffer (20 mM Tris+HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, and 2 mM EDTA) containing protease inhibitor cocktail, and 300 μ g protein was incubated overnight at 4°C with 4 μ g goat polyclonal anti-Chk1 antibody (R&D Systems); 50 µl protein A/G agarose was added and incubated for 2 hours at room temperature. Beads were washed three times with IP buffer. Immune complex was eluted by adding 1× SDS loading dye and heating at 100°C, and immunoblotting was performed to detect STAT3, pSTAT3 (Y705 or S727), and Chk1 (mouse monoclonal; Cell Signaling Technology). Images were captured using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

Real-Time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen Corporation) as per the manufacturer's protocol. Forward and reverse primer sequences for specific genes or promoters were designed using the MacVector software (MacVector, Inc., Cary, NC) and are listed in Table 2.

MTT Assay

Five thousand HPTECs were plated in a 96-well plate for 24 hours in DMEM/F12 with supplements or DMEM supplemented with 10%

FBS. Cells were then treated with HU and incubated for 24 hours; 1 mg/ml MTT was added, and it was further incubated for 2 hours. Medium was aspirated, and 100 μ l isopropanol was added. Absorbance was measured at 570 nm taking 630 nm as reference using SpectraMax Paradigm (Molecular Devices, Sunnyvale, CA). Absorbance obtained from untreated cells was taken as 100% (*n*=12 wells/ treatment), and the experiments were repeated three times.

ChIP Assay

ChIP was performed using the Magna ChIP G Tissue Kit as per the manufacturer's instructions (EMD Millipore, Billerica, MA) with some modifications. Approximately 3-mm diameter nonfixed snap frozen kidney tissues were used, and DNA was crosslinked with protein by adding 900 μ l freshly diluted 1% formaldehyde/protease inhibitor in PBS. A number of washing, centrifugation, and sonication steps were performed to get DNA fragments ranging from 200 to 1000 bp. Samples were spun at 10,000×g at 4°C for 10 minutes, and 1500 μ g protein was used in 500 μ l volume for IP with 10 μ g STAT3, normal mouse IgG (negative control), or RNA polymerase (positive control) antibodies. Fifteen micrograms (1%) protein from each sample was frozen in 80°C as input. Protein G magnetic beadsantibody/chromatin complex were separated by magnetic rack and washed with 500 µl low-salt immune complex wash buffer, high-salt immune complex wash buffer, LiCl immune complex wash buffer, and tris-ethylenediaminetetraacetic acid buffer. One hundred microliters ChIP elution buffer/proteinase K was added in IP as well as input samples and incubated at 62°C for 2 hours with shaking, and proteinase K was denatured by heating at 95°C for 10 minutes. For IP samples, protein G magnetic beads were separated by a magnetic separator rack, and supernatant was used for DNA purification. Five hundred microliters bind reagent A was added to each DNA sample tube (IP and inputs). Sample/bind reagent A was transferred to the spin filter and centrifuged for 30 seconds at $10,000 \times g$; 500 μ l wash reagent B was added to the spin filter and centrifuged at $10,000 \times g$ for 30 seconds. Spin filter was dried by centrifuging again at 10,000 \times g for 30 seconds and placed in a clean microfuge tube, and DNA was eluted by applying 50 μ l elution buffer C directly onto the spin filter followed by incubation for 30 seconds and centrifugation at

Table 2. List of primer pairs used fo	r RT-PCR
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Gene	Sequence			
cDNA-specific primers used				
for RT-PCR				
Rat KIM-1				
Forward	5'-TAT TTG GGG GAA CAG GTT GC			
Reverse	5'-CAA GTC ACT CTG GTT AGC CGT G			
Human KIM-1				
Forward	5'-CTG CAG GGA GCA ATA AGG AG			
Reverse	5'-TCC AAA GGC CAT CTG AAG AC			
Promoter-specific				
primers used for				
ChIP assay				
Rat KIM-1				
Forward	5'-GGG AGT CCT TGA GTA TTT GGT GC			
Reverse	5'-ATG TTT GCT CGG CTT CCT GG			

 $10,000 \times g$ for 30 seconds. Five-microliter samples were used for real-time PCR using promoter-specific primers of KIM-1 (Table 2).

Luciferase Assay

Semiconfluent (70%-80%) HPTECs were transfected with pRC/ CMV or pRC/CMV_STAT3 using DharmafectDuo transfection reagent (as per the manufacturer's instruction), washed 6 hours post-transfection, and incubated for 24 hours. Cells were then cotransfected with pRL-GAPDH (gift from Dr. Matthias Brock, Center of Experimental Rheumatology, University Hospital Zurich, Zurich, Switzerland) and KIM-1 luciferase wild-type or mutant constructs, washed 6 hours post-transfection, and further incubated for 24 hours. Semiconfluent (70%-80%) HPTECs were cotransfected with pRL-GAPDH and KIM-1 luciferase wild-type or mutant constructs, washed 6 hours post-transfection, treated with vehicle, IL-6, and dexamethasone or HU, and further incubated for 24 hours. Semiconfluent (70%-80%) 769-P cells were transfected with Ctrl or STAT3 siRNA using Dharmafect1 transfection reagent (as per the manufacturer's instruction), washed 6 hours post-transfection, and further incubated for 48 hours. Cells were then cotransfected with pRL-GAPDH and KIM-1 luciferase wild-type or mutant constructs, washed 6 hours post-transfection, and further incubated for 24 hours. Cells were then lysed by passive lysis buffer, and luminescence was measured using the Dual-Glo Luciferase Assay System (Promega Corporation) on a Veritas Luminometer (Turner Biosystems, Sunnyvale, CA). KIM-1 Luciferase readings (Firefly luciferase) were normalized to GAPDH Luciferase (Renilla luciferase) readings, and fold change was calculated compared with Ctrl HPTECs or Ctrl 769-P cells.

Immunostaining, Confocal Imaging, Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-Deoxyuridine Nick-End Labeling, and Phase Contrast Imaging

Immunostaining in human kidney patient tissue sections, HPTECs, or 769-P cells was performed using pSTAT3 (Y705 or S727) and pChk1 or KIM-1 (gift from Dr. Joseph V. Bonventre, Brigham and Women's Hospital, Boston, MA) antibodies. Rat kidney sections were immunostained with pH2A.X antibodies. The primary antibody was detected using species-specific Cy3- and FITC-labeled secondary antibodies (Jackson ImmunoResearch Laboratories); 4',6-diamidino-2-phenylindole (Sigma-Aldrich) was used for nuclear staining. Image was captured by either a Nikon DS-Qi1Mc camera attached to a Nikon Eclipse 90i fluorescence microscope using an oil immersion objective 60/1.4 numerical aperture by Nikon NIS elements AR version 3.2 software or a Carl Zeiss AxioImager.M2 using AxioVision SE64 software by a 63×/1.4 oil DIC objective. We used a Zeiss LSM710 confocal microscope, and the image was captured by Zen software using a 63×/1.4 oil DIC objective. DNA damage was measured in kidney tissues or HPTECs by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling assay using the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. The images were captured on a Carl Zeiss AxioImager.M2 using AxioVision SE64 software by $63 \times /1.4$ oil DIC objective. For phase contrast imaging, cells were treated with indicated concentrations of HU and imaged using Spot advanced software on a Nikon Eclipse TE2000-E using a Plan Fluor $10 \times / 0.30$ objective.

Computational Analyses

We used the gene expression profiles representing the time-scaled measurements of transcript levels in rat kidney cortex and medulla associated with the induction of IRI (measured at the time points of 0, 6, 24, and 120 hours after the injury; $n=3/\text{time point}^{23,24}$; Gene Expression Omnibus accession number GSE27274). The expression profile was obtained from rat kidney (cortex and medulla, respectively) after 0, 6, 24, and 120 hours after IRI. We used hierarchical clustering to identify a subset of probes (n=220) that showed a substantial upregulation in early time points after the injury (6 and 24 hours) in both cortex and medulla. For clustering, 1-Pearson correlation was used as the distance with average linkage. A total of 174 gene symbols was obtained after removing the redundancy of 220 probes, and 174 genes were measured for the enrichment with known targets of transcription factors using ChEA analysis.²¹ The ChEA database contains the experimentally validated targets of transcription factors (290 targets sets of various transcription factors). Top significant enrichment was considered as the potential regulation of the corresponding transcription factors. As an additional filter, we measured the IRI-associated expression level of the selected transcription factors and selected three transcription factors (MYC, KLF4, and STAT3) that showed substantial upregulation associated with IRI in cortex and medulla. We also used ARACNe software to identify the additional targets of the three transcription factors of MYC, KLF4, and STAT3. We used bioinformatics tools to identify upstream regulators such as kinases. First, Genes2Networks²⁷ software was used to identify the interaction partners of the selected transcription factors. Second, the selected transcription factors and their interacting partners were measured for the enrichment with known kinase substrates and targets using KEA analysis.²² Top significant enrichments were considered as potential regulatory functions of the corresponding kinases in the kidney IRI.

Statistical Analyses

Data are expressed as average + SEM. Statistical difference (P<0.05) was calculated by *t* test. *P*<0.05 was considered significant and represented by an asterisk compared with controls. All graphs were generated by GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

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DISCLOSURES

None.

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