Mammalian Target of Rapamycin Mediates Kidney Injury Molecule 1-Dependent Tubule Injury in a Surrogate Model

Wenqing Yin,* Said Movahedi Naini,* Guochun Chen,* Dirk M. Hentschel,* Benjamin D. Humphreys,*† and Joseph V. Bonventre*†‡

*Renal Division, Brigham and Women’s Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts; †Harvard Stem Cell Institute, Cambridge, Massachusetts; and ‡Division of Health Sciences and Technology, Harvard-Massachusetts Institute of Technology, Cambridge, Massachusetts

ABSTRACT

Kidney injury molecule 1 (KIM-1), an epithelial phagocytic receptor, is markedly upregulated in the proximal tubule in various forms of acute and chronic kidney injury in humans and many other species. Whereas acute expression of KIM-1 has adaptive anti-inflammatory effects, chronic expression may be maladaptive in mice. Here, we characterized the zebrafish Kim family, consisting of Kim-1, Kim-3, and Kim-4. Kim-1 was markedly upregulated in kidney after gentamicin-induced injury and had conserved phagocytic activity in zebrafish. Both constitutive and tamoxifen-induced expression of Kim-1 in zebrafish kidney tubules resulted in loss of the tubule brush border, reduced GFR, pericardial edema, and increased mortality. Kim-1-induced kidney injury was associated with reduction of growth of adult fish. Kim-1 expression led to activation of the mammalian target of rapamycin (mTOR) pathway, and inhibition of this pathway with rapamycin increased survival. mTOR pathway inhibition in KIM-1-overexpressing transgenic mice also significantly ameliorated serum creatinine level, proteinuria, tubular injury, and kidney inflammation. In conclusion, persistent Kim-1 expression results in chronic kidney damage in zebrafish through a mechanism involving mTOR. This observation predicted the role of the mTOR pathway and the therapeutic efficacy of mTOR-targeted agents in KIM-1-mediated kidney injury and fibrosis in mice, demonstrating the utility of the Kim-1 renal tubule zebrafish models.


Kidney injury molecule 1 (KIM-1), also known as T cell Ig and mucin 1 or hepatitis A virus cellular receptor-1 (HAVCR1), is a type 1 transmembrane protein. In the normal mammalian kidney, KIM-1 expression is undetectable, but after acute injury its expression is induced abundantly in the proximal tubules, where it localizes to the apical surface of epithelial cells and also to the basolateral membrane when polarity is lost.† Acting as a nonmyeloid phosphatidylserine (PS) receptor, the mammalian KIM-1 ectodomain binds and internalizes oxidized lipids as well as PS exposed on the outer surface of luminal apoptotic cells.‡–¶ KIM-1 clears the tubule lumen of debris following AKI, aiding in nephron repair and tissue remodeling. KIM-1 is also upregulated in a variety of animal models of CKD, including protein overload nephropathy,§ adriamycin-induced nephropathy,‖ angiotensin II-induced renal damage,‖ and murine polycystic kidney disease,ʰ where it colocalizes with areas of fibrosis and inflammation.‖ It is upregulated in human CKD and its expression correlates directly with interstitial fibrosis in human allografts.¹¹

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Correspondence: Dr. Joseph V. Bonventre, Renal Division, Harvard Institute of Medicine, 4 Blackfan Circle, Boston, MA 02115. Email: joseph_bonventre@hms.harvard.edu

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Recently, we reported a transgenic mouse model with nephron-specific overexpression of KIM-1. These mice develop tubular damage, reduced nephron number, and fibrosis, and have a high mortality rate compared with controls. These observations suggested a pathogenic role of KIM-1 in linking AKI to CKD and suggested KIM-1 as a novel therapeutic target in CKD. Embryonic overexpression of KIM-1 in these transgenic mice, however, resulted in reduced nephron numbers, raising the possibility that this might have contributed to the CKD phenotype.

Studies of CKD require intact organ systems to recapitulate the interplay of the multiple pathophysiologic processes involved in chronic inflammation and fibrosis; however, mice or other mammalian models are not suitable for high-throughput screening of therapeutic agents, due to the high cost and large time requirements of the screening. The zebrafish (Danio rerio), a small tropical freshwater fish, has become an excellent vertebrate model for studying human disease, genetics, and development. Despite the anatomic simplicity of the zebrafish pronephros, and a range of ciliopathies have brought throughputs, high degree of structural and functional homology with mammalian models is not suitable for high-throughput screening for therapeutic drug discovery.

In this study, using phylogenetic and genomic analysis, we characterized the zebrafish KIM-1 gene family, which consists of k1, k3, and k4. Zebrafish KIM-1 protein shares a high degree of structural and functional homology with mammalian KIM-1. Similar to what is observed in mammals, zebrafish KIM-1 expression was markedly upregulated in the kidney tubules after injury. Kidney tubular cell expression of KIM-1 resulted in pathologic effects in the pronephros and mesonephros, and associated inhibition of fish growth in size. KIM-1-induced nephrotoxicity is inhibited by mTOR inhibition with rapamycin. This finding is recapitulated in the mouse overexpressing KIM-1 in the renal tubule, thus validating the zebrafish model as a surrogate for KIM-1-induced CKD in the mouse.

RESULTS

Identification of Zebrafish Kim-1 Family

By searching public protein and cDNA databases (European molecular biology laboratory [EMBL], Genbank, and University of California Santa Cruz [UCSC]), and using the National Center for Biotechnology Information (NCBI)/BLAST program, we identified the k1 (EMBL accession number ENSDARG00000091692), k3 (ENSA00000077257), and k4 (ENSDARG00000040178) genes in zebrafish. Zebrafish KIM-1 has a putative signal peptide (SP) of 19 amino acids (aa), an Ig domain of 105 aa, a mucin domain of 172 aa, a transmembrane domain of 20 aa, and a cytoplasmic domain of 44 aa. Comparison of identity and similarity of each domain of zebrafish KIM-1 (zKIM-1), zebrafish KIM-3 (zKIM-3), and zebrafish KIM-4 (zKIM-4) with their human and mouse orthologs, revealed that the zKIM-1 Ig domain has the highest score of identity (approximately 40%) and similarity (approximately 57%) with mouse KIM-1 (mKIM-1) and human KIM-1 (hKIM-1) (Figure 1A). There was also a high degree of homology with the highest identity and similarity score between zKIM-3, zKIM-4, and their human orthologs in the Ig domain (hKIM-3 and hKIM-4) (Supplemental Figure 1, A and B).

Phylogenetic analysis placed zebrafish k1 in the same branch as human KIM-1, alongside other mammalian KIM-1s (Figure 1B). Analysis of the genomic location of the zebrafish k1 family, based on zv9 assembly, demonstrated that all three genes are located on chromosome 21, adjacent to each other, although the positional order of k1, k3, and k4 on the chromosomes is not conserved between zebrafish chromosome 21 and human chromosome 5 (Figure 1, C and D). To identify possible splice variants of k1 in zebrafish, we performed 3′ and 5′ rapid amplification of cDNA ends (RACE). k1 has three splice variants. k1-L is the longest splice variant and contains all exons except exon 4 (Figure 1D). k1-S is the short splice variant and lacks exon 4 and 6. k1-Ig is an Ig domain splice variant, which consists only of the Ig domain and incomplete mucin domain (exons 1–6).

Expression Pattern of Kim-1, Kim-3, and Kim-4 in Zebrafish After Injury

Kidney injury in mammals is associated with a marked upregulation of KIM-1 expression in proximal tubular cells. We previously reported a gentamicin-induced proximal tubular injury model in the zebrafish. To examine the expression of KIM-1 in zebrafish after injury, adult zebrafish (>90 days postfertilization [dpf]) were injected with gentamicin (5 μg/100 mg body wt). Quantitative RT-PCR was used to define the relative expression patterns for each k family member at 2 days after gentamicin injection (Figure 2). After gentamicin-induced injury, k1-L and k1-S mRNA expression were higher in kidney compared with other organs, while k3 and k4 were expressed at low levels in all organs including the kidney. Immunofluorescence staining for KIM-1 was performed with a specific antibody that we produced against the Ig domain of KIM-1 (Supplemental Figure 2). Lotus tetragonolobus lectin (LTL) (proximal tubules marker) and Dolichos biflorus agglutinin (DBA; distal tubules and collective ducts marker) were used as markers of nephron segments. Kim-1 was expressed on the apical membrane of proximal tubular cells but not distal tubular or collecting duct cells after gentamicin-induced kidney injury (Figure 3A). The time-course of expression of k1 mRNA revealed that k1-L and k1-S transcripts in adult zebrafish kidney were upregulated by 2 days postgentamicin injection (dpi), and remained elevated until 8 dpi, after which they decreased to near-normal.

levels at 12 dpi, while the expression of *kim1*-Ig or *kim3* and *kim4* transcripts was not altered by gentamicin administration (Figure 3B). In larvae, the expression of *kim1*-L and *kim1*-S transcripts were also upregulated at 8 dpf after gentamicin was administered at 6 dpf (Supplemental Figure 3).

**Zebrafish Kim-1 Is a Phagocytic Receptor for Apoptotic Bodies and oxidized-LDL**

As a PS receptor, mammalian KIM-1 confers on proximal tubular cells the ability to recognize PS on apoptotic cells and mediates the phagocytosis of apoptotic cells, necrotic cells, and oxidized LDL (ox-LDL). The binding motif WFND has been known to be essential for the phagocytic function of KIM-1. The alignment of zKIM-1 with mKIM-1 and hKIM-1 protein sequences demonstrated that the FND residues are conserved, while the tryptophan (W) residue is replaced with leucine (L) (Figure 3C). The WFND motif is completely conserved in zKim-4. Similar to mammalian KIM-3, there is lack of conservation of the WF residues in zKim-3 (Figure 3C).

To determine whether zebrafish Kim-1 has phagocytic activity, *kim1*-L was cloned from gentamicin-treated zebrafish larvae. LLC-PK1 cells were transfected with expression plasmids encoding zebrafish *kim1*-L fused to EGFP (zkim1-EGFP) or human KIM-1 fused to EGFP (hKIM-1-EGFP), and then incubated with Dil-labeled apoptotic thymocytes or ox-LDL for 2 hours (Figure 3D). Immunofluorescence staining...
showed engulfment of apoptotic thymocytes or ox-LDL (white arrowheads) by zKim-1-expressing cells (zKim-1-EGFP) and hKIM-1-expressing cells (hKIM-1-EGFP). To quantify the phagocytic capacity of zebra fish Kim-1, LLC-PK1 cells transfected with plasmids encoding EGFP, zkim1-EGFP, or hKIM-1-EGFP were cocultured for 2 hours at 37°C with fluorescent Dil-labeled apoptotic thymocytes. Undigested apoptotic cells were then washed away and live cells were lifted into a single-cell suspension with EDTA and trypsin. Two-color flow cytometry showed that, in hKIM-1-EGFP-PK1 and zKim-1-EGFP-PK1 cells, the number of phagocytosed apoptotic thymocytes was approximately 10-fold greater than cells expressing EGFP alone (51.5% ± 0.33% were positive for apoptotic cells in hKIM-1-EGFP-PK1 versus 53.5% ± 0.32% in zKim-1-EGFP-PK1 versus 5.89% ± 0.24% in EGFP-PK1) (Figure 3E). These data demonstrate that zebrafish Kim-1 is a phagocytic receptor for apoptotic cells and ox-LDL, indicating a conserved phagocytic function between zebra fish Kim-1 and the human ortholog.

Figure 2. Tissue distribution of Kim family genes in adult zebrafish after injury. Quantification of kim1 (three forms, kim1-lg, kim1-l, and kim1-S), kim3 and kim4 mRNA expression in liver, pancreas, fin, intestine, eye, heart, brain, muscle, and kidney at 2 days after gentamicin injection relative to controls with saline injection (quadruplicate, n=12 for each group). *P<0.05; **P<0.01. For all tissues, gene expression level was normalized to β-actin and relative to the corresponding tissues from saline-injected control fish, using the ΔΔCt method.

Overexpression of Kim-1 in Pronephric Kidney Causes Renal Failure in Zebrafish

The cadherin 17 (cdh17) promoter was used to overexpress Kim-1 in kidney tubular epithelial cells in vivo. We generated a pTol-cdh17:Kim1-RFP vector, encoding kim1-L fused with the RFP reporter (kim1-RFP). The pTol-cdh17:RFP plasmid was generated for comparison. Zebrafish eggs were injected with kim1-RFP or RFP-expressing constructs at the one-cell stage. At 48–72 hours after injection, we carefully selected fish that expressed Kim-1-RFP or RFP predominantly in the tubule, excluding those with significant nontubule (gut) expression. Approximately 100 RFP-positive fish were analyzed in each group. Kim-1-RFP-expressing fish began to develop pericardial edema at 2 dpf, which became more prominent at 3 dpf, compared with RFP-expressing fish and control fish (wild-type fish without injection) (Figure 4, A–C). Kim-1-RFP protein expression was confirmed by Western blot analysis (Figure 4D). Kim-1-RFP-expressing fish had a much higher incidence of pericardial edema at 3 dpf (47.9% ± 3.15% versus 5.5% ± 0.93% in RFP-expressing fish and 0.53% ± 0.35% in uninjected control fish) (Figure 4E). Survival was lowest in those fish expressing Kim-1 (Figure 4F).

To evaluate the functional consequences of Kim-1 overexpression, the clearance of 10 kDa dextran was measured to determine GFR in zebrafish.15 We injected wild-type larvae (control), and larvae expressing RFP or Kim-1-RFP with 2 ng tetramethylrhodamine-labeled 10 kDa dextran at 72 hours postfertilization (hpf) (0 hours) (Figure 4G). Fluorescence intensities were measured over the eye at 1, 7, 24, 31, and 43 hours after injection and compared with the baseline (immediately upon equilibration after injection, 0 hours). The renal clearance of tetramethylrhodamine-labeled 10 kDa dextran was significantly delayed in Kim-1-overexpressing fish, indicating reduced GFR (Figure 4H).
Persistent Expression of Kim-1 in Tubular Cells Results in Chronic Kidney Damage in Zebrafish

Because of genetic mosaicism caused by partially penetrant expression of the cdh17 promoter, in some of the transgenic zebrafish Kim-1 expression was detectable in only one of the two pronephric tubules (Supplemental Figure 4). To examine the effect of chronic expression of Kim-1 on the mesonephros, surviving transgenic larvae overexpressing Kim-1-RFP in one pronephric tubule were raised to adulthood. By 12 weeks of age, these fish were of smaller size and lower weight compared with age-matched RFP-expressing controls (Figure 5, A–F). Hematoxylin and eosin (H&E) staining of whole fish revealed smaller kidney parenchyma in Kim-1 transgenic zebrafish, compared with RFP reporter controls. Persistent Kim-1 expression was verified by immunofluorescence staining (Figure 5B). Dissection of kidneys, followed by histologic examination, revealed that Kim-1-overexpressing fish had fewer glomeruli, commensurate with reduced kidney area, but a normal density of glomeruli (Figure 5, E and F). Periodic acid–Shiff (PAS) staining and electron microscopy (EM) demonstrated tubular damage in Kim-1-overexpressing cells with loss of brush border and accumulation of vacuoles by light microscopy and phagosomes by EM (Figure 5, G and H).

Cre/LoxP Mediated Conditional Expression of Kim-1 in the Pronephros Causes Tubular Damage in Zebrafish

Because the fully functional glomerular barrier in zebrafish is formed at 72–96 hpf,22 it is possible that transgenic Kim-1 expression results in uptake of noxious factors from the glomerular filtrate that are not normally present in the tubule lumen after the glomerulus has fully matured. To address this possibility, we created a conditional Cre-mediated and tissue-specific overexpression model of Kim-1, in which Kim-1 is expressed in differentiated epithelial tubular cells after maturation of the glomerular filtration barrier. We first generated a stable transgenic zebrafish line expressing Cre-ERT2 in nephrons under control of the cdh17 promoter. The

**Figure 3.** Kim-1 is upregulated after gentamicin-induced injury and is a phagocytic receptor for apoptotic bodies in zebrafish. (A) Kidney injury was induced by gentamicin injection in adult zebrafish (>90 dpf). Immunofluorescence staining with markers for proximal tubule (LTL, green), distal tubule (DBA, white on the top and purple on the bottom), nuclei (4′,6-diamidino-2-phenylindole [DAPI], blue) and anti–Kim-1 antibody (red) in mesonephric kidneys at 2 and 6 dpi, shows that Kim-1 is expressed on the apical side of proximal tubular (PT) cells (white arrowhead) but not DBA-positive distal tubular (DT) cells. (B) Quantitative PCR was performed at various time points on isolated entire kidneys. There is a significant increase in Kim1-long (Kim1-L) and Kim1-short (Kim1-S) mRNAs at 2 dpi, with maximum expression at 6 dpi. The expression returns closer to normal levels at 12 dpi (triplicate, n=15 for each group). All quantitative data are expressed as mean±SEM. **P<0.01. Kim1-Immunoglobulin domain (Kim1-Ig); (C) Alignment of the PS binding motif WFND in zebrafish and human Kim families. In zKim-1, W (tryptophan) has been replaced by L (leucine), while the four residues are conserved in zKim-3, zebra

schematic of this construct is shown in Figure 6A. To verify this transgenic line we crossed it with the ubi:loxP-EGFP-loxP-RFP line. Addition of 4-hydroxytamoxifen (4-OHT) at 4 dpf showed a robust expression of RFP in pronephric tubules, while addition of the vehicle, ethyl alcohol (EtOH), resulted in no expression of RFP (Figure 6B). The excision efficiency of the cdh17:Cre-ERT2 transgenic zebrafish line was high in each segment of the tubule (Supplemental Figure 5). Next, we generated a ubi:loxP-EGFP-loxP-Kim-1-RFP transgenic zebrafish, in which floxed-EGFP is driven by the ubiquitin promoter (ubi) (Figure 6C). Addition of 4-OHT results in Cre-mediated excision of EGFP and expression of Kim-1-RFP. One cell stage embryos from cdh17:Cre-ERT2 transgenic zebrafish were injected with 25 ng of tetramethylrhodamine-labeled 10 kDa dextran, and serial fluorescence images were quantitated over the eye at 0, 1, 7, 24, 31, and 43 hpi (n=20 fish per condition). Data are expressed as mean±SEM. *P<0.05.

Figure 4. Kim-1 overexpressing zebrafish larvae develop pericardial edema, renal failure, and have a higher mortality. (A–C) Bright field and RFP fluorescence images of uninjected zebrafish (control) and fish injected with Tol2(cdh17:RFP) or Tol2(cdh17:kim1-RFP) vector at the one-cell stage. Tol2(cdh17:kim1-RFP) zebrafish begin to develop detectable edema at 2 dpf. Black arrows indicate the edema area; white arrows indicate RFP or Kim-1-RFP expression in kidney tubules (scale bar, 50 μm). (D) Western blot analysis of RFP expression in Tol2(cdh17:RFP) or Tol2(cdh17:kim1-RFP) zebrafish. (E, F) Tol2(cdh17:RFP) or Tol2(cdh17:kim1-RFP) zebrafish were analyzed daily for edema formation and survival rate (the total number of tested fish is indicated). (G, H) At 96 hpf, wild-type (control) or Tol2(cdh17:RFP) or Tol2(cdh17:kim1-RFP) larvae were injected with 25 ng of tetramethylrhodamine-labeled 10 kDa dextran, and serial fluorescence images were quantitated over the eye at 0, 1, 7, 24, 31, and 43 hpi (n=20 fish per condition). Data are expressed as mean±SEM. *P<0.05.

The Activation of mTOR in Kim-1 Constitutive Transgenic Zebrafish
We used our Kim-1 constitutive transgenic zebrafish model to gain insight into mechanisms of injury associated with Kim-1 expression and to identify potential therapeutic agents. At 3 months, the activation of caspase 3 was increased in Kim-1 constitutive transgenic zebrafish, consistent with increased tubular cell apoptosis associated with chronic Kim-1 overexpression (Figure 7A). The percentage of caspase 3-positive tubular cells was markedly increased in proximal (LTL+) and distal (DBA+) as well as LTL−/DBA+ regions of Kim-1 expressing transgenic zebrafish when compared with non-Kim-1 expressing controls (Figure 7B).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a pivotal role in mediating cell size
and mass, proliferation, and survival. We examined the activation of mTOR and evaluated whether inhibition of the mTOR pathway with rapamycin could protect against Kim-1-mediated kidney injury. As shown in Figure 7C, phosphorylated ribosomal S6 kinase (pS6K), the downstream target of mTOR, was increased in Kim-1 constitutive transgenic zebrafish at 3 months of age. pS6K localized to the apical surface of tubular cells that also expressed Kim-1 (Figure 7C). Compared with RFP-expressing controls, an increased number of pS6K-positive tubular cells were found in LTL+, DBA+, and LTL−/DBA− regions of Kim-1 transgenic zebrafish (Figure 7D).

Rapamycin Reduced Pericardial Edema and Mortality in Zebrafish Conditionally Overexpressing Kim-1

Expression of Kim-1 in the tubular epithelial cells of conditional transgenics was induced at 5 dpf. Rapamycin (50 nM) was added into the E3 medium at 9 dpf and maintained in the media after the addition. Time-course live images show that the development of both pericardial and yolk sac edema in tamoxifen-induced Kim-1 transgenic fish was reversed by rapamycin treatment at 10 dpf and 12 dpf (Figure 8A). Quantification of relative area of pericardial and yolk edema demonstrated that rapamycin treatment significantly reduced Kim-1-mediated pericardial and yolk edema formation (Figure 8B). To test the effects of rapamycin treatment on long-term mortality, conditional Kim-1 expression was induced at 5 dpf followed by addition of rapamycin at 5, 7, 9, or 11 dpf. Rapamycin was maintained in the media after the addition. These conditional Kim-1 transgenics died spontaneously at a median age of 21 days without rapamycin treatment, while rapamycin treatment of Kim-1 transgenic zebrafish significantly reduced mortality, with earlier treatment associated with more protection (Figure 8C).

Rapamycin Treatment in KIM-1 Transgenic Mice Ameliorated Serum Creatinine and Kidney Inflammation, and Diminished Fibrosis

Next, we sought to examine whether this zebrafish model could predict mTOR pathway involvement in the pathophysiology of transgenic mice where KIM-1 is expressed in the kidney tubule derived from the Six-2+ metanephric mesenchyme (Kim1RECtg mice). The expression levels of mTOR pathway protein mRNAs were increased progressively in Kim1RECtg mice at 2 and 4 weeks of age (Figure 9A). Enhanced levels of pS6K were present in Kim1RECtg mice at 5 weeks (Figure 9B). Quantification of the number of pS6K-positive cells showed increased number in Kim1RECtg mice at 5 weeks but not at 2 weeks of age, when compared with age-matched controls (Figure 9C). To test whether inhibition of mTOR by rapamycin protects against kidney injury and reduces the development of kidney fibrosis, Kim1RECtg mice...
were treated with rapamycin by daily intraperitoneal (ip) injection (2 mg/kg per day, n=6) or saline (n=6) starting at 4 weeks of age for up to 6 weeks and mice were examined at 10 weeks of age. Staining with trichrome and PAS showed decreased kidney fibrosis, tubule dilatation, interstitial expansion, and interstitial mononuclear cell infiltration (Figure 9D).

The tubule injury scores and fibrosis index were lower in rapamycin-treated Kim1RECtg mice (P<0.05 and P<0.01 respectively; Figure 9E). Serum creatinine was lower and proteinuria less in rapamycin-treated mice at 10 weeks (P<0.01, Figure 9, F and G). In Kim1RECtg mice treated with rapamycin, there was a significant reduction in the number of the Ly-6G+ neutrophils; F4/80+ peritubular macrophages and dendritic cells (Figure 10A); Ki67+ tubular, glomerular, and interstitial proliferating cells; and CD3+ lymphocyte infiltration (Figure 10, B and C). Rapamycin decreased expression of pS6K, aSMA, fibronectin, and collagen-1 in Kim1RECtg mice (Figure 10, D–F). The mRNA levels of cortical cytokines, CXCR3, CXCL-10, IL-1β, TNF-α, CXCL-1, IL-6, CXCL-2, MCP-1, and the marker of inflammation NGAL, were reduced (Figure 10G). Ly-6G+, collagen-1, and fibronectin mRNAs were also significantly reduced in Kim1RECtg mice treated with rapamycin (Figure 10, H–J).

DISCUSSION

KIM-1 is upregulated in a variety of human diseases and animal models, and is specifically expressed in injured proximal tubular epithelial cells, making it a sensitive and specific marker of proximal tubule injury in the kidney. In this study, we first identified and characterized the Kim/Tim family of proteins in zebrafish. Similar to mammalian KIM-1, zebrafish Kim-1 was markedly and specifically upregulated at the apical aspect of proximal tubular cells following kidney injury. The phagocytic function was also conserved in zebrafish Kim-1.

To evaluate whether chronic expression of Kim-1 resulted in kidney tubular injury in zebrafish, we generated transgenic zebrafish models in which Kim-1 was constitutively or conditionally expressed in the pronephric or mesonephric tubular epithelial cells. The tamoxifen-inducible conditional Kim-1 transgenic line was created to avoid potential early developmental consequences of Kim-1 expression. In both constitutive and conditional transgenic zebrafish, there was kidney tubular injury, reduced renal function, and systemic consequences including edema, smaller size, and an increased mortality rate.
Figure 7. Increased activation of caspase 3 and S6K in Kim-1 constitutive transgenic zebrafish. (A) Immunofluorescence staining of active caspase 3 (green) in Kim-1 constitutive transgenic zebrafish and controls at 3 months of age. 4',6-diamidino-2-phenylindole (DAPI; blue) (scale bar, 50 μm). (B) Quantification of active caspase 3-positive cells in different nephron regions of Kim-1-RFP or RFP control zebrafish (n=5, ten sections for each). The proximal tubules and distal tubules were distinguished by LTL and DBA expression, respectively. (C) Immunofluorescence staining of pS6K (green) in Kim-1-RFP or RFP control fish at 3 months of age, DAPI (blue), (scale bar, 50 μm). (D) Quantification of active pS6K-positive cells in different nephron regions of Kim-1 transgenics or RFP controls (n=5, ten sections for each). Data are expressed as mean±SEM. *P<0.05; **P<0.01.

These data indicate that zebrafish Kim-1 expression can model mammalian chronic KIM-1 expression and hence enable mechanistic studies of the consequence of KIM-1 expression using the power of genetic manipulation. Furthermore, given that zebrafish are amenable to high-throughput drug screening, these transgenic lines will be useful to identify inhibitors of Kim-1 and potentially effective therapies for CKD. As proof of principle, we used this zebrafish model to identify rapamycin as a protective agent in zebrafish and confirmed that this protection could also be seen in the mouse model of KIM-1 overexpression.

Acute tubular damage can facilitate the development and progression of CKD, which is associated with interstitial fibrosis in human and other mammalian species.66 Although temporary expression of KIM-1 after injury will help to eliminate the apoptotic cells and debris and partially restore renal function, persistent KIM-1 expression in renal epithelial cells induces inflammation, tubulointerstitial fibrosis, and a murine CKD phenotype.37 Here, we have demonstrated that persistent expression of Kim-1 in zebrafish tubular epithelial cells results in the development of pericardial edema, reduced GFR, tubular damage, and a higher mortality. The damaged pronephric tubules were marked with flattening and loss of tubular brush border and atrophic tubular epithelial cells with accumulation of vacuoles.

Compared with the zebrafish pronephros, the zebrafish mesonephros more closely resembles the human metanephros. Prolonged Kim-1 expression in mesonephric tubular epithelial cells led to a lower body weight and reduced overall zebrafish size. As zebrafish age, new nephrons are continually added to the kidneys.16,38 Persistent expression of Kim-1 in the mesonephros resulted in smaller kidneys. The smaller kidneys and reduced growth of zebrafish are reminiscent of the growth retardation associated with renal disease in children and suggests a permissive role of an increase in nephrons and kidney function for growth in size of fish.

To evaluate the effects of Kim-1 expression independent of any potential effects on development or glomeruli barrier maturation, we utilized a tamoxifen-induced conditional Kim-1 overexpression model in the pronephros. These Kim-1 transgenic zebrafish develop phenotypes similar to those seen with constitutive expression: pericardial and yolk sac edema and increased mortality rates.

mTOR, a serine/threonine kinase, is activated after AKI and has been implicated in renal regeneration and repair.29 In addition, the activation of mTOR also occurs in a variety of animal models of diabetic nephropathy and other progressive CKD.39–41 We hypothesized that KIM-1-induced injury may involve mTOR pathway activation, which could contribute to kidney fibrosis and progressive CKD. In the zebrafish, we observed that the expression of kim-1 was associated with mTOR activation, and that the mTOR inhibitor, rapamycin, protected against kim-1-induced injury. We then confirmed the power of the zebrafish model by demonstrating the protective effect of rapamycin translated to KIM-1-mediated kidney tubular injury in Kim1RECtg mice, where rapamycin treatment significantly reduced the kidney fibrosis and interstitial inflammatory response. In our Kim-1 transgenic zebrafish and mice models, mTOR activity was highly associated with Kim-1 expression. Kim-1 is known to directly interact with phosphatidylinositol-3-kinase (PI3K) pathway subunit p85 and regulate PI3K signaling in a phosphotyrosine-dependent manner.32 Recently, we demonstrated that Kim-1...
Figure 8. mTOR inhibitor, rapamycin, reduced edema and mortality of conditional Kim-1 transgenic zebrafish. (A) Time-course live images show, compared with EtOH controls, that 4-OHT-induced Kim-1 expression in tubules resulted in pericardial edema, which was ameliorated by RAP treatment. RAP expression marks Kim-1 expression. The black stars mark the edema areas. The black arrow indicates ameliorated edema. Inset shows the expression of Kim-1-RFP in nephrons (scale bar, 50 μm). (B) Quantification in two dimensions of area of pericardial and yolk sac edema in both 4-OHT-induced Kim-1 transgenic zebrafish with or without RAP at 9 dpf relative to EtOH vehicle-treated fish. The areas of pericardial and yolk edema were analyzed with ImageJ software (n=60 for each group). Data are expressed as mean±SEM. *P<0.05; **P<0.01. (C) Mortality associated with 4-OHT-induced Kim-1 expression in both kidney tubules is mitigated by RAP addition. The effect of RAP is greater when it is given earlier. The total number of tested zebrafish in each condition is listed.

interaction with p85 and subsequent PI3K-dependent signaling pathway play an important role in kidney injury and repair. The PI3K/AKT/mTOR pathway is a well known intracellular signaling pathway related to cell growth, proliferation, and survival. Thus, it is likely Kim-1 directly binds to p85 and regulates mTOR activity through the AKT signaling pathway.

In conclusion, our study is the first to identify the Kim family genes in zebrafish and to characterize the function and expression of Kim-1 after injury. Kim-1 can potentially be used as a marker for tubular injury in zebrafish, as in rodents and humans. Conditional expression of Kim-1 in the transgenic zebrafish demonstrated a critical pathogenic role of Kim-1 in chronic kidney tubular injury. Kim-1 expression markedly reduced kidney function and fish survival, and retarded fish growth in the adults that did survive. We found that the mTOR pathway was activated in Kim-1 transgenic zebrafish and inhibition of this pathway significantly reduced Kim-1-mediated kidney injury and increased fish survival. We extended the zebrafish findings into mammalian models by examining the Kim-1-mediated mTOR pathway in mice and found that it was activated in Kim1RECtg mice. Inhibition of the mTOR pathway significantly ameliorated the Kim-1-mediated tubular injury and kidney fibrosis in Kim1RECtg mice. Thus, the zebrafish models can be used to interrogate pathophysiologic mechanisms and potential therapeutic approaches which predict mechanisms and efficacy in mammals.

CONCISE METHODS

Structural and Molecular Evolutionary Analyses
Homology analysis with nucleic acid and protein databases (Genbank, EMBL, and SwissProt), including human expressed sequence tag databases, was performed using the BLAST algorithm from the NCBI (National Library of Medicine). Aa analysis for putative post-translation modification sites was performed with PROSITE. Ig domains were predicted by the PROSITE database. The transmembrane domain was predicted using the online program “TMpred” (www.ch.embnet.org/software/TMPREDDform.html), and O-linked glycosylation sites were predicted by the NETNGLYC 3.1 server (www.cbs.dtu.dk/services/NetOGLyc). The draft genome databases and expressed sequence tag databases distributed at Swiss-Prot protein databases, Expasy, Ensembl, University of California, Santa Cruz (UCSC) Genome Browser, The Institute of Genomic Research (TIGR), and NCBI database for Expressed Sequence Tags (dbEST) were employed to retrieve the Ig molecules. Multiple alignments of sequences were conducted using the Multi-Align software and Clustal W program (version 1.83). On the basis of the alignment, phylogenetic trees were constructed with the program MEGA using a neighbor-joining method. Statistical significance of each branch was examined by bootstrapping. The veracity of these trees was studied using the bootstrapping method by executing 1000 replicates.

Zebrafish Husbandry
All animal husbandry adheres to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Wild-type zebrafish (D. rerio) were maintained on a 14/10 hour light–dark cycle at 28.5°C and fed twice daily. Fertilized eggs were raised in embryo medium at 28.5°C and staged according to the standard method as...
Figure 9. Rapamycin treatment ameliorated kidney injury and fibrosis in Kim1<sup>REC</sup>tg mice. (A) Real-time PCR demonstrates increased mRNA expression of mTOR pathway intermediates in Kim1<sup>REC</sup>tg mice relative to controls at 2 and 4 weeks. *P<0.05; **P<0.01 (n=6 for each). (B) The immunofluorescence staining of pS6K in Kim1<sup>REC</sup>tg and control mice at 2 and 5 weeks of age. (C) Quantification of the number of pS6K-positive cells in Kim1<sup>REC</sup>tg or controls **P<0.01 (n=6 for each). (D) Trichrome and PAS staining of control or RAP-treated Kim1<sup>REC</sup>tg mice. At 4 weeks of age mice were given daily ip injection of RAP (2 mg/kg per day) for 6 weeks (n=6). Kidneys were taken at 10 weeks of age. Kidney fibrosis, tubule dilation, interstitial expansion, and interstitial mononuclear cell infiltration were decreased after RAP treatment of Kim1<sup>REC</sup>tg kidneys (scale bar, 50 µm). (E) Quantification of the tubule injury score and fibrosis index in Kim1<sup>REC</sup>tg after vehicle or RAP treatment (n=6 for each). (F) Serum creatinine at 4 weeks and 10 weeks of age in control (n=6) or Kim<sup>REC</sup>tg mice (n=6), treated with vehicle or RAP. Data are expressed as mean±SEM. *P<0.01; ***P<0.001. (G) Coomassie blue staining of control or RAP-treated Kim1<sup>REC</sup>tg mouse kidneys at 10 weeks of age shows the reduction of proteinuria with RAP treatment. DAPI, 4',6-diamidino-2-phenylindole.

Described previously<sup>13</sup>. All the eggs and larvae were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, 10–5% methylene blue).

Induction of AKI by Gentamicin and Renal Function Measurements

For the gentamicin and dextran-FITC injection, zebrafish larvae were anesthetized in a 1:20–1:100 dilution of 4 mg/ml tricaine (Sigma-Aldrich) and positioned on their back in a 1% agarose injection mold. Using a Nanoject II injection device (Drummond Scientific, Broomall, PA), 2.3 nl of a 10 mg/ml gentamicin (Sigma-Aldrich) stock solution and 5 ng of 40 kDa dextran-FITC (Molecular Probes), to confirm drug delivery, were injected into the cardiac venous sinus. Fish were then returned to egg water, where they regained motility quickly. Renal function was assayed as previously described.<sup>12</sup> Briefly, 25 ng of tetramethylrhodamine-labeled 10 kDa dextran was injected into the cardiac venous sinus of control or Kim-1-overexpressing larvae at 72 hpf. With constant exposure time and gain, images were taken at 1, 7, 24, 31, and 43 hpi. More than ten fish were studied in each group. The fluorescence intensities over the eye were analyzed using the NIH ImageJ software. For adult zebrafish, gentamicin (5 µg/100 mg) and 40 kDa dextran-FITC (25 ng) were administered by ip injection.

Transgenic Zebrafish

To generate the pTol-cdh17:kim1-RFP construct, the zebrafish <i>kim1</i>-<i>L</i> coding sequence was cloned by RT-PCR. It was fused to the RFP coding sequence (kim1<sup>L</sup>-RFP) and was inserted in place of the EGFP gene in the pTol-cdh17:EGFP construct (gift from Weibin Zhou, University of Michigan), using KpnI and NotI restriction enzymes. For the pTol-cdh17:RFP control, we replaced EGFP with RFP at BamH1 and NotI sites. To generate the <i>ubi:loxP-EGFP-loxP-kim1-RFP</i> construct, <i>ubi:loxP-EGFP-loxP</i> sequence was cloned from the <i>cdh17:EGFP</i> construct (gift from Leonard I. Zon, Harvard Medical School), and inserted into PminiTol2 plasmid at EcoRV and SpeI sites. Then the Kim1<sup>L</sup>-RFP sequence was inserted at SpeI and XhoI sites. For creating the <i>cdh17:Crem-ERT<sup>T2</sup></i> construct, <i>Crem-ERT<sup>T2</sup></i> (a gift from Dr. Ben Humphreys, Brigham and Women’s Hospital) replaced the EGFP gene in the pTol-cdh17:EGFP construct at KpnI and NotI sites. All plasmid DNAs listed above were injected into one-cell stage embryos. These injected embryos were raised and the fish were screened for Kim-1 and/or RFP expression. To generate the conditional Cre-mediated and nephron-specific Kim1 transgenic zebrafish, <i>pTol-ubi:loxP-EGFP-loxP-kim1-RFP</i> plasmids were injected into Tg(cdh17:Crem-ERT<sup>T2</sup>) stable line offspring.

Rapamycin Treatment

In zebrafish, rapamycin (50 nM) was added into the E3 medium at 5, 7, 9, or 11 dpf and maintained in the media after the addition. In mice, the Kim1<sup>REC</sup>tg mice were treated with rapamycin by daily ip injection (2 mg/kg per day) or saline starting at 4 weeks of age for up to 6 weeks and mice were examined at 10 weeks.

Tissue Preparation and Histology

Zebrafish were euthanized in 300 mg/L tricaine. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned and stained with H&E or PAS using standard procedures. For EM, kidneys were harvested, fixed with 2.5% glutaraldehyde, and sectioned.
Kidney area was measured on H&E-stained sections of five kidneys in each group by ImageJ software. Glomeruli in these sections were counted blinded to the genotype. The number of glomeruli per area of kidney was determined. The two-dimensional area of pericardial and yolk sac edema were measured on live images of fish in each group. Areas were quantitated using ImageJ software.

Mice were anesthetized, euthanized, and immediately perfused via the left ventricle with ice-cold PBS for 2 minutes. Kidneys were hemisectioned, and portions were snap-frozen in liquid nitrogen. Other kidneys were fixed in 10% neutral buffered formalin at 4°C for 12 hours, processed, embedded in paraffin wax, sectioned, and stained with PAS using standard procedures. PAS-stained paraffin sections were assessed by quantitative measurement of tubular injury in ten individual high-power fields (magnification, ×400) per kidney. A percentage of the area affected was estimated for the number of necrotic cells, loss of brush border, cast formation, and tubule dilation and was scored as follows: 0, 0%–5%; 1, 5%–10%; 2, 11%–25%; 3, 26%–45%; 4, 46%–75%; and 5, >76%. The fields analyzed in each section were selected at random and evaluations were made in a blinded fashion.

Zebrafish Kim-1 Antibody

From the zebrafish Kim-1 full protein sequence, we designed three anti-peptide antibodies against LQLNYRESHRFS, LYLISEKMTTDDVRM, and LFLRLRRYREQTI derived from Ig domain, mucin domain, and cytoplasmic domain, respectively. Antibodies were synthesized by Genemed Synthesis Inc. (San Antonio, Texas). Western Blot analysis (Supplemental Figure 2) demonstrates that the expression of Kim-1 was upregulated in gentamicin-treated adult zebrafish and hypoxia-exposed larval zebrafish, compared with wild-type adult fish and...
larval *kim1* morphants (*kim1* Mo, GTGCAATTAAACACTCAGATTCTTC) (Supplemental Figure 2). The anti- *Kim-1* antibody (1:100) raised against the Ig domain peptide was used throughout the manuscript for immunofluorescence staining.

**Immunofluorescence Staining**

Cryosections of 5 μm were mounted on Fisher Superfrost Plus microscope slides (Thermo Fisher Scientific), air-dried and prepared for immunofluorescence. LTL (lectin, 1:500, cat. no. L-1030; Vector Laboratories) and DBA (fluorescein, 1:500, cat. no. L-1321; Vector Laboratories) were used to identify proximal and distal tubules in zebrafish and mice. Primary antibodies against the following proteins were used: cleaved caspase 3, pS6K, fibronectin, and collagen I (rabbit polyclonal, 1:100, Cell Signaling Technology); RFP and Na\(^+\)/K\(^+\) ATPase V9 (mouse monoclonal, 1:100, Abcam, Inc.); F4/80 (rat, 1:100, cat. no. 6640; Abcam, Inc.); SMA (mouse FITC coupled, 1:100, cat. no. F3777, Sigma-Aldrich); Ki67 (rabbit monoclonal, 1:1000, cat. no. VP-RM01; Vector Laboratories); and Ly-6G (rat monoclonal, 1:200, cat. no. F3777, Sigma-Aldrich); Ki67 (rabbit monoclonal, 1:100, cat. no. 6640; Abcam, Inc.); SMA (mouse FITC coupled, 1:200, cat. no. VP-RM04; Vector Laboratories); and Ly-6G (rat monoclonal, 1:200, cat. no. F3777, Sigma-Aldrich). Secondary antibodies were obtained from Dako. Sections were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Images were taken with a Nikon TE2000 microscope (Roper Scientific) and processed using Nikon NIS Elements software. Immunofluorescence images were obtained on a Nikon TE2000 or a Nikon C1 Eclipse confocal microscope using standard procedures. For the quantification, percentages of active caspase 3 and pS6K-positive tubular cells were scored manually by counting the number of cells stained with the anti-active caspase 3 and anti-pS6K antibodies. The samples were randomized prior to examination, and the person performing the counting was not aware of the experimental conditions. The proximal and distal tubules were distinguished with LTL and DBA staining. The total number of tubular cells was determined by scoring the number of nuclei (stained with 4',6-diamidino-2-phenylindole). Thirty different fields (40× magnification) were randomly taken from kidney sections and analyzed as described above.

**Histologic Analysis and EM**

Histologic analysis was performed on paraffin-embedded fish using 3-μm sections, stained with H&E or PAS. Portions of zebrafish larvae were fixed in Karnovsky fixative and processed for EM studies by standard procedures. Semithin sections of each block were stained with toluidine blue stain and examined by light microscopy to select for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by EM.

**Western Blot Analysis**

Kidneys were isolated and lyzed as previously described.\(^3\) Membranes were incubated with rabbit anti- *Kim-1* peptide antibodies against LQLNYRESHRSF (antibody #1) and LFLRLRRYEQTI (antibody #3) (1:500, Genemed Synthesis Inc.); mouse anti-RFP (1:200, Clontech); mouse anti-αSMA (1:200, Abcam, Inc.); pS6K (1:500; Cell Signaling Technology); and ERK (1:1000; Cell Signaling), followed by goat anti-mouse HRP (1:200, Jackson ImmunoResearch). Bands were visualized by chemiluminescence (Western Lightning; PerkinElmer).

**Expression Analysis of Genes by Quantitative RT-PCR**

Total RNA was isolated from snap-frozen kidney, brain, heart, eye, fin, intestine, pancreas, muscle, gills, and liver of five adult zebrafish with RNeasy Kit. Of total RNA, 1 μg was treated with DNase RQ1 (Promega) and then reverse transcribed with the M-MLV reverse transcription Kit and Oligo dT primers (Promega). Real-time, quantitative RT-PCR was performed using a BioRad iCycler. Samples were in triplicate, and all experiments were repeated three times using separately prepared samples. Statistical analyses was performed using SPSS 11 software. Statistical significance for quantitative RT-PCR was analyzed by the Mann–Whitney U test. PCR was conducted with specific primers for the following mRNAs: *kim1* F: AACAATTGCGCCCCACAAACAG, R: CGGACATCATCTGTTGCTATCAT; *kim-1*-Ig F: TGATCGCTGGCTGATCAG, R: ATCCAGGCAGAACTCACA; *kim1*-L F: TGTACGTCTGGCCTATCGAG, R: GGCTTCTGAGCAAAGTTTGTGTTT; *kim1*-S F: TGTAACGTCTGCGATCAG, R: CGGACATCTGTTGCTATCATC; *kim3* F: ATTCGCCGTTGGTTCTCTGTGC, R: AATGTGTCT-GGCTCCTTTTG; *kim4* F: TATCCCCAGTAA-GGGGCTTG, R: ATCTCCAGTGGCTGATGTCG; β-actin F: GTGACCATCTACGAGGTATTTA, R: TCTACGAGGTGTTGATGAAAG; PISK F: CCAGGACCATCCTGCGGT; ACGAGGACATCTAAGTACTA; AKT F: ATGAACAGCAGTGACATTGG; TGTAGGCA-AAAGGCTGCAT; mTOR F: ACCGGAGCACTTGAAGAG; CRTC F: CTGGTGAGGATCATCAAG; 4EBP1 F: GGGGACATCACGAC-CTC; R: CTCGGTGAGGATGACAG; elF4E-BP3 F: CCTGCGGCGACACTATACG; R: GTATCTGCGGAGAGTGTGA; P70S6 F: GCTTGGTGATGCGGGATGTTT; R: GCTGATGCTTCAC-GACCTAT; CXCL1 F: CTGGGATCCTACACAAGCCTAC; CAGGGTCAAAGGAACGCTC; CXL2 F: CCAACACACAGGGCTAGG; GCGTCAGAATGACTGCT; CXL10 F: CCA-ATGTGCGGCTCACTT; GGCCTGAGGATGTACCTAC; CLEC1 F: CTTCTGAGGATGACAG; AAGCTCACACAG-CAGGAG; IL-1β F: CCCTCCAGGATGACAG; R: AAGCTTACACAG-CAGGAG; IL-6 F: TATGCTTCTTACCCCAATTCC; R: TGGTGCTTACGACTCCTCC; MCP-1 F: TGATCTGCGCC-TAAGTCTTC; R: AAGTGTGCTAGTGGTTGAGTT; TGF-β F: GCAAAATTCCTGGGTTCATT; R: CGAAACCTTGATTCCTGCT; TNF-α F: CCCTCACACTCAGATCTCCT; R: GCTAC-GAGTGCGGTACAG.

**Phagocytosis Assays**

To quantitate phagocytosis, hKIM-1-GFP-PK1, zKim-1-GFP-PK1, and GFP-PK1 cells (3×10^5 each) were plated in 3.8 cm^2 wells 24 hours prior to assay. 1×10^6 Dil-labeled apoptotic thymocytes or 50 mg/ml ox-LDL was added to confluent epithelial cell layers. After 2 hours at 37°C, cells were washed vigorously five times with ice-cold PBS/0.1% sodium azide (without Mg^2+ /Ca^2+ ) to remove bound and uningested apoptotic cells or debris. Cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100 and blocked with 0.1% BSA. Primary and appropriate secondary antibodies were applied to the cells, and imaging was performed with a Nikon confocal microscope.
After incubation with apoptotic cells, the cells were washed, trypsinized, and stained with primary anti-EGFP antibody and FITC-conjugated secondary antibody, resuspended in FACS buffer and subjected to flow cytometry (FACSCalibur; BD Biosciences). Ingestion of Dil-labeled apoptotic cells was identified using green and red channels. Data were analyzed by FlowJo software version 9.4.7.

**Statistical Analyses**

Results were calculated as mean±SEM. Analysis of variance was used to compare data among groups. Two-tailed, unpaired t tests were used to compare two groups. P values <0.05 (⁎P<0.05 and **P<0.01) were considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**


reference genome sequence and its relationship to the human ge-
Yamazaki S, Enomoto Y, Oki T, Akiba H, Abe T, Komori T, Monikawa Y,
Kiyonari H, Takai T, Okumura K, Kitamura T: TIM1 is an endogenous
ligand for LMR5/CD300b: LMR5 deficiency ameliorates mouse kidney
22. Kramer-Zucker AG, Wiensner S, Jensen AM, Drummond JA: Organi-
zation of the pronephric filtration apparatus in zebrafish requires
Nphrin, Podocin and the FERM domain protein Mosaic eyes. Dev Biol
285: 316–329, 2005
tous transgene expression and Cre-based recombination driven by the
24. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR,
Choi Y, Gray NS, Yaffe MB, Marta JO, Sabatini DM: The mTOR-regulated
phosphoproteome reveals a mechanism of mTORC1-mediated inhibition
H, Tempst P, Sabatini DM: mTOR interacts with raptor to form a nutrient-
sensitive complex that signals to the cell growth machinery. Cell 110:
163–175, 2002
B: SIN1/MITP1 maintains rictor-mTOR complex integrity and regulates Akt
27. Wullschleger S, Loewith R, Hall MN: TOR signaling in growth and
28. Hresko RC, Mueckler M: mTOR.RICTOR is the Ser473 kinase for Akt/
protein kinase B in 3T3-L1 adipocytes. J Biol Chem 280: 40406–40416,
2005
29. Lieberthal W, Fuhr W, Andry CC, Rennke H, Abemathy VE, Koh JS,
Valer R, Levine JS: Rapamycin impairs recovery from acute renal failure:
role of cell-cycle arrest and apoptosis of tubular cells. Am J Physiol Renal
Physiol 281: F693–F706, 2001
30. Johnson SC, Rabinovitch PS, Kaeberlein M: mTOR is a key modulator of
parmcy prevents early steps of the development of diabetic ne-
32. Llobetras N, Cruzado JM, Franquesa M, Herrero-Fresnedal I, Terras J,
Alperovich G, Rama I, Grinyó JM: Mammalian target of rapa-
mycin pathway blockade slows progression of diabetic kidney disease
attenuates unilateral ureteral obstruction-induced renal fibrosis. Kid-
ney Int 69: 2029–2036, 2006
34. Diekmann F, Rovira J, Carreras J, Arellano EM, Bañón-Maneus E,
Ramírez-Bajo MJ, Gutiérrez-Dalmay A, Brunet M, Campistol JM:
Mammalian target of rapamycin inhibition halts the progression of
proteinuria in a rat model of reduced renal mass. J Am Soc Nephrol 18:
2653–2660, 2007
16: 556–561, 2010
cell cycle arrest in G2/M mediates kidney fibrosis after injury. J Nat Med
16: 535–543, 2010
37. Yang L, Brooks CR, Xiao S, Sabbisetti V, Yeung MY, Hsiao LL, Ichimura
T, Kuchroo V, Bonventre JV: KIM-1-mediated phagocytosis reduces
38. Zhou W, Boucher RC, Bollig F, Englert C, Hildebrandt F: Character-
ization of mesonephric development and regeneration using trans-
Dai C: Rheb/mTORC1 signaling promotes kidney fibroblast activation
F, Cordts T, Wanner N, Reichardt W, Kerjaschki D, Ruegg MA, Hall MN,
Moulin P, Busch H, Boerries M, Walz G, Artunc F, Huber TB: mTORC1
maintains renal tubular homeostasis and is essential in response to is-
Rictor/mTORC2 protects against cisplatin-induced tubular cell death
42. de Souza AJ, Oak JS, Jordanhazy R, DeKruyff RH, Fruman DA, Kane LP:
T cell Ig and mucin domain-1-mediated T cell activation requires
recruitment and activation of phosphoinositide 3-kinase. J Immunol
180: 6518–6526, 2008
43. Brooks CR, Yeung MY, Brooks YS, Chen H, Ichimura T, Henderson JM,
Bonventre JV: KIM-1/TIM-1-mediated phagocytosis links ATG5/ULK1-
dependent clearance of apoptotic cells to antigen presentation [pub-
lished online ahead of print August 17, 2015]. EMBO J doi:1015252/
embj.201489838
44. Loh AH, Brennan RC, Lang WH, Hickey RJ, Malkas LH, Sandoval JA:
Dissecting the PI3K signaling axis in pediatric solid tumors: Novel tar-
gets for clinical integration. Front Oncol 3: 93, 2013
45. Vanhaesebroeck B, Stephens L, Hawkins P: PI3K signalling: the path to
46. Hofmann K, Bucher P, Falquet L, Bairoch A: The PROSITE database,
47. Falquet L, Pagni M, Bucher P, Hofmann K, Sigrist CJ, Bairoch A:
238, 2002
48. Copet F: Multiple sequence alignment with hierarchical clustering.
49. Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the
sensitivity of progressive multiple sequence alignment through se-
quence weighting, position-specific gap penalties and weight matrix
50. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGAS:
molecular evolutionary genetics analysis using maximum likelihood,
evolutionary distance, and maximum parsimony methods. Mol Biol Evol
28: 2731–2739, 2011
This article contains supplemental material online at http://jasn.asnjournals.
Fig. S1. Alignment of zebrafish Kim-3 and Kim-4 family protein sequences with human and mouse orthologues. (A) Protein sequence of zebrafish Kim-3 (zKim-3) was aligned with mouse and human orthologues (mKIM-3, hKIM-3), horizontal red lines indicate the Ig-like domain (Ig). (B) Protein sequence of zebrafish Kim-4 (zKim-4) was aligned with mouse and human orthologues (mKIM-4, hKIM-4). Horizontal red lines indicate the Ig-like domain (Ig).
Fig. S2. Analysis of the specificity of Kim-1 antibody. Western Blot analysis of Kim-1 expression in adult zebrafish after gentamicin injury, wild-type or 96hpf larvae with hypoxia or kim1 morpholino injection, using two different anti-Kim-1 peptide antibodies (Antibody #1, A or #3, B), respectively. The red rectangles outline the bands that react with the antibodies at the predicted size of Kim-1 in zebrafish.
Fig. S3. Kim-1 is upregulated after gentamicin-induced injury in larvae. Zebrafish larvae were injected with gentamicin at 6dpf. qPCR was performed at 8dpf. There is a significant increase in *kim1-L* and *kim1-S* mRNA at 2d post gentamicin injection (n=3, 5 fish/group). Data are expressed as mean±SEM. ** P<0.01
Fig. S4. Mosaicism of Kim-1 expression in transgenic zebrafish. (A) H&E staining of cross section of a 1 week old larvae overexpressing Kim-1 in one of the two tubules of the pronephros, scale bar, 50 µM. B) Inset shows the two nephrons. Black arrow points to the Kim-1 expressing tubule. C-E) Na⁺/K⁺ ATPase, RFP and merged expression in subsequent section of tissue. Blue, DAPI staining. scale bar, 20 µM.
Fig. S5. The excision efficiency of the cdh17:Cre-ER\textsuperscript{T2}, ubi:loxP-EGFP-loxP-RFP transgenic zebrafish line crossed to ubi:loxP-EGFP-loxP-RFP transgenic fish. (A) Excision was induced by 4-OHT in cdh17:Cre-ER\textsuperscript{T2} and ubi:loxP-EGFP-loxP-RFP double transgenic fish at 4dpf. Immunofluorescence staining of cross sections of different nephron regions for EGFP and RFP shows the loss of EGFP and gain of RFP expression in nephrons at 7dpf. PCT (proximal convoluted tubule), PST (proximal straight tubule), DE (distal early), DCT (distal convoluted tubule), CD (collecting duct). The dashed lines indicate the level of the nephron where the section is taken. Rectangles and inserts mark the zebrafish nephrons, (scale bar, 50 µm). (B) Quantification of the percentage of cells that switched from EGFP to RFP in different nephron regions, (n=3, 4 for each). Data are expressed as mean ± SEM.