MDM2 prevents spontaneous tubular epithelial cell death and acute kidney injury

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Murine double minute-2 (MDM2) is an E3-ubiquitin ligase and the main negative regulator of tumor suppressor gene p53. MDM2 has also a non-redundant function as a modulator of NF-κB signaling. As such it promotes proliferation and inflammation. MDM2 is highly expressed in the unchallenged tubular epithelial cells and we hypothesized that MDM2 is necessary for their survival and homeostasis. MDM2 knockdown by siRNA or by genetic depletion resulted in demise of tubular cells in vitro. This phenotype was completely rescued by concomitant knockdown of p53, thus suggesting p53 dependency. In vivo experiments in the zebrafish model demonstrated that the tubulus cells of the larvae undergo cell death after the knockdown of mdm2. Doxycycline-induced deletion of MDM2 in tubular cell-specific MDM2-knockout mice Pax8rtTa-cre; MDM2f/f caused acute kidney injury with increased plasma creatinine and blood urea nitrogen and sharp decline of glomerular filtration rate. Histological analysis showed massive swelling of renal tubular cells and later their loss and extensive tubular dilation, markedly in proximal tubules. Ultrastructural changes of tubular epithelial cells included swelling of the cytoplasm and mitochondria with the loss of cristae and their transformation in the vacuoles. The pathological phenotype of the tubular cell-specific MDM2-knockout mouse model was completely rescued by co-deletion of p53. Tubular epithelium compensates only partially for the cell loss caused by MDM2 depletion by proliferation of surviving tubular cells, with incomplete MDM2 deletion, but rather mesenchymal healing occurs. We conclude that MDM2 is a non-redundant survival factor for proximal tubular cells by protecting them from spontaneous p53 overexpression-related cell death.

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Results

MDM2 prevents tubular epithelial cell death in vitro. The knockdown (KD) of MDM2 in mouse tubular epithelial cell line with siRNA resulted in decreased viability of tubular cells in
comparison to tubular cells treated with control siRNA (Figure 1a). MDM2 suppression was associated with a marked increase in p53 and p53 effector genes p21 and PUMA transcript levels (Supplementary Figure 1A). The concomitant KD of MDM2 and p53 significantly improved the viability of tubular epithelial cells in vitro (Figure 1a).
This result was confirmed in primary tubular cells MDM2 KO pTECs isolated from Pax8rtTA-cre; MDM2f/f mice, where MDM2 was depleted specifically in tubular epithelial cells in vitro by treatment with doxycycline. The generation of these mice is described below. MDM2 mRNA levels decreased significantly in MDM2 KO pTECs treated with 1μg doxycycline (Figure 1b). The Mdm2-deficient primary tubular cells showed increased expression of tubular damage markers KIM-1, NGAL and TIMP-2 as well as increased cell death, due to the upregulation of p53 (Figure 1b). Increased p53 activity was confirmed by elevated mRNA expression of p53-target genes p21 and PUMA (Supplementary Figure 1B). The simultaneous depletion of MDM2 and p53 completely rescued the viability of the primary tubular cells (Figure 1b).

The pTECs population was about 95% pure as assessed by staining for the tubular epithelial cell markers cytokeratin-7 and E-cadherin (Figure 1c). To prove the specificity of MDM2 depletion in tubular epithelial cells, we isolated also parietal epithelial and mesangial cells from kidneys of Pax8rtTA-cre; MDM2f/f, treated them with doxycycline, and checked mRNA levels of MDM2 and viability of those cells. Unlike in primary tubular cells, MDM2 expression and viability remained unchanged in the glomerular cells in comparison to the controls (Supplementary Figure 1C). Ultrastructural analysis of murine primary tubular cells lacking MDM2 revealed vacuolization of cytoplasm, nicks in the cytoplasmic membrane, and eventually cell death with complete disassembly of the cell structure (Figure 1d). These results suggest that MDM2 is required to protect the tubular cells from p53 overexpression-related cell death.

Mdm2 KD in zebrafish larvae induces cell death of renal tubular epithelial cells. To validate the in vitro results in vivo, a KD of mdm2 was generated in zebrafish larvae by the injection of specific morpholinos into fertilized eggs and was confirmed by qRT-PCR (Figure 2a). At 4 days post fertilization (d.p.f.), the mdm2 KD zebrafish larvae developed pericardial edema (white arrow in Figure 2b), a hallmark of kidney failure, in contrast to control morpholino-injected larvae (Ctrl) and larvae with a double KD knockout of mdm2 and p53 (mdm2/p53). For a rapid screening of the larvae (Ctrl) and larvae with a double KD knockout of mdm2 and p53 (mdm2/p53), we used the transgenic zebrafish strain Tg(wt1b:eGFP) that expresses eGFP in podocytes, parietal epithelial cells and proximal tubules. Sections of zebrafish larvae revealed a significant dilatation of the pronephric tubules in mdm2 KD larvae (Figure 2c) which could be rescued to some extent by the KD of p53. Additionally, we have found less epithelial cells in the proximal tubules after the mdm2 KD compared with Ctrl and mdm2/p53 KD larvae (Figure 2c). The TUNEL assay demonstrated that this is due to an increase of dead cells in mdm2 KD larvae (Figure 2d). Furthermore, the remaining cells showed a reduced expression of the Na+-K+-ATPase, a marker of differentiated tubular epithelial cells (Figure 2c).

In vivo imaging of the pronephros of living Tg(wt1b:eGFP) larvae by two-photon microscopy (2-PM) confirmed the significant dilatation of the Bowman space after mdm2 KD (asterisk in Figure 2e; Supplementary Movie 1) in contrast to mdm2/p53 and Ctrl KD larvae (Supplementary Movies 2 and 3). We have found that the maximum width of the pronephric proximal tubules in living zebrafish larvae significantly increased from 7.03 μm (S.E.M. = 2.29 μm, n = 7) in Ctrl and 8.53 μm (S.E.M. = 2.19 μm, n = 7, P = 0.01) in mdm2/p53 KD larvae to 14.67 μm (S.E.M. = 2.42 μm, n = 9, P = 0.044) in mdm2 KD larvae (Figure 2f). These results show that mdm2 is essential for pronephric tubular epithelial cells by protecting them from a p53-dependent cell death.

Mdm2 depletion in tubular epithelium results in acute kidney injury. To confirm these findings in the mammalian kidney, we crossed Pax8rtTA-cre mice with MDM2f/f mice to generate a mice model with inducible MDM2 deletion exclusively in renal tubular epithelial cell upon doxycycline treatment, Pax8rtTA-cre; MDM2f/f (Figure 3a). These mice were born at expected Mendelian ratios and did not show any functional or renal histological abnormalities within 6 months of age. Real-time PCR analysis of kidney extracts from Pax8rtTA-cre; MDM2f/f mice treated with doxycycline (MDM2−/− tubulus) for 4, 8 or 11 days demonstrated progressively declining levels of MDM2 mRNA compared with control mice (Figure 3b). MDM2 immunostaining was selectively diminished in the renal tubular cells of the Pax8rtTA-cre; MDM2f/f mice treated with doxycycline (MDM2−/− tubulus) while the MDM2 immunostaining remained intact in the glomerulus, confirming thus the selectivity of the MDM2 tubular deletion (Figure 3c; Supplementary Figure 2A). MDM2−/− tubulus mice showed progressive impairment of kidney function in comparison to control mice, as documented by significant increase of plasma creatinine and blood
urea nitrogen (BUN) levels (Figure 3d). Measured glomerular filtration rate (GFR) declined from day 4 of doxycycline treatment in MDM2−/− tubulus mice and at day 8 the mice were severely oliguric compared with control littermates (Figure 3e). The decline of MDM2−/− tubulus mice kidney function was also associated with the shortened lifespan.

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The MDM2 deleterious phenotype in tubular epithelium is p53 dependent. To test for the role of p53 in the structural and functional renal pathology of MDM2−/− tubulus mice, we generated inducible tubular epithelial cell-specific simultaneous MDM2 and p53 knockout mice MDM2/p53KO tubulus and the littermate controls with one p53 allele intact MDM2−/− tubulus /p53wt. We treated the both mouse lines with doxycycline to induce the deletion of the respective gene alleles. The analysis of the functional and histological parameters of the mice models confirmed that the depletion of p53 rescues the pathological phenotype of the MDM2−/− tubulus mice (Figures 6a–e).

Tubular epithelium compensates only partially for the cell loss caused by MDM2 depletion. Previously, it was reported that mice lacking Mdm2 in the intestinal epithelium can fully compensate for the MDM2 depletion/p53 activation-mediated cellular loss because of negative selection of the MDM2 transgenic cells and rapid proliferation and overgrowth of cells with insufficient Cre recombinase activity and thus incomplete MDM2 deletion. To investigate whether this phenomenon occurs also in renal tubular epithelium, which is known for its fast regenerative capacity, we subjected the Pax8rtTA-cre; MDM2fl/fl mice to different doxycycline treatment regimens to avoid the rapid lethality of the continuous doxycycline treatment. We treated the mice with doxycycline for 2 days to deplete the MDM2 just in the portion of the tubular epithelial cells and repeated the treatment after 5 days of recovery for three times. The renal function of the experimental mice was impaired compared with the control mice as documented by elevated serum creatinine and BUN levels (Figure 7a) but the lifespan of the experimental animals was not affected (data not shown). The histological analysis showed focal tubular damage with overproduction of extracellular matrix, suggesting that the loss of tubular cells is only partially recovered by surviving tubular cells but rather mesenchymal repair occurs (Figure 7b). Masson trichrome staining confirmed the accumulation of the fibrotic tissue in the kidneys of experimental mice (Figure 7b) and also mRNA expression levels of several pro-fibrotic genes were elevated (Figure 7c). We detected moderate p53 activation with nuclear shift throughout the tubular compartment with especially high expression in medullar region of the kidney (Figure 7b). We conclude that unlike intestinal epithelium, tubular epithelial cells cannot completely compensate for the cells lost due to the MDM2 deletion.
Discussion

We hypothesized that MDM2 is important for homeostasis of resting tubular epithelial cells in kidney. In the cell culture experiments the tubular epithelial cells lacking MDM2 showed decreased viability. The mouse model with the specific deletion of MDM2 in tubular epithelial cells displayed shortened lifespan, fast decline in renal function as
documented by reduced GFR and increased plasma creatinine and BUN. We also detected significant upregulation of tubular damage markers and massive swelling, mitochondrial degeneration and cell death in the tubular compartment in the kidneys of the experimental animals. All these changes developed in unchallenged kidney and were consistent with acute tubular injury. The tubular epithelium was not able to fully compensate for the cell loss due to the MDM2 depletion by proliferation of remaining tubular cells with intact MDM2 but rather mesenchymal healing with fibrosis occurred. The pathological phenotype was completely rescued by co-deletion of p53. We conclude that MDM2 is essential for survival and homeostasis of intact tubular cells in healthy kidney.

MDM2, the main negative regulator of p53 suppresses its function in three different ways; it chaperons p53 out of the nucleus, that is, out of the transcriptional centrum, it blocks the transcription of p53 effector genes and it ubiquitinates p53 and targets it for proteasomal degradation. Through the p53 inhibition, MDM2 fosters cell proliferation and prevents p53-mediated cell death, cell cycle arrest and senescence. This MDM2 function is indispensable for the embryonic development, wound healing and regeneration but it also facilitates autoimmune disorders, such as lupus erythematosus and carcinogenesis. MDM2 is overexpressed frequently in many human tumors, especially in breast cancer and sarcomas. The inhibitors of MDM2 and p53 binding are being currently tested in clinical trials as potential cancer therapy. Nevertheless also MDM2 depletion has deleterious effects as documented by embryonic lethality of a mouse model with genetic-deletion of MDM2. The mouse embryos die due to the uncontrolled p53-dependent cell death, while p53 deficiency completely rescues this deleterious phenotype.

Specific deletion of MDM2 in developing kidney results in impairment of renal progenitor cell expansion, in aberrant nephrogenesis and differentiation and leads to hypoplasia of the kidneys. Our data are in line with these studies and show that MDM2 is crucial for the survival and homeostasis of unchallenged tubular epithelial cells in kidney. MDM2 prevents p53-mediated cell death of these renal cells. Our findings differ from a study which showed that unbuffered p53 activity caused by MDM2 deletion is detrimental only in radiosensitive tissues due to the massive cell loss, while the radio-insensitive tissues, including kidney, are protected from cell death but exhibit inhibition of cell proliferation. Our data indicate that MDM2 depletion in tubular epithelial cells leads to massive cell death due to p53 overactivation but proliferation activity is maintained as documented by increased expression of Ki-67, marker of proliferation. Our results are corroborated by another study showing that global MDM2 deletion results in morphological and functional abnormalities in both radiosensitive and radio-insensitive tissues, including kidney. Renal phenotype included initially just mild damage with no aberrant functional effect, but subsequently the kidneys exhibited severe injury with impairment of renal function. Further on, MDM2 deletion in intestinal epithelial cells results in multiple intestinal abnormalities in newborn animals. These abnormalities completely disappeared in adulthood due to the selection against the enterocytes lacking MDM2 and increased proliferation of the epithelium with intact MDM2. Although the renal tubular epithelium has an extensive regenerative capacity, our findings indicate that in kidneys of tubular specific depleted MDM2 mice the complete epithelial healing does not occur and is compensated by mesenchymal healing with extensive fibrosis. This suggests that the inhibition of MDM2 could lead to acute kidney injury and due to incomplete healing contribute to development of chronic kidney disease. In our previous studies we showed that MDM2 in acute kidney injury promotes tubular injury in early postischemic phase via augmentation of NF-kB signaling and thus inflammation in p53-independent manner. Our present data document, that all renal anomalies due to MDM2 depletion in unchallenged tubular epithelial cells are dependent on p53 activity, as the defects completely disappeared when p53 was absent. This is in line with studies which showed dose-dependent attenuation of tubular cell death and kidney injury in mouse model of cisplatin induced nephrotoxicity treated with p53 siRNA or with chemical inhibitor of p53.

Together, MDM2 is crucial in intact tubular epithelial cells in kidney to prevent spontaneous p53 overactivation dependent cell death and thus prevent acute tubular injury. As MDM2 antagonists are being developed as an alternative treatment to chemotherapy for cancer treatment, it is of note that MDM2 inhibition might be detrimental for normal tissues, especially for kidney as our data suggest. We conclude that MDM2 is essential for survival and homeostasis of tubular epithelium in kidney.

Materials and Methods
Cell culture experiments. Murine tubular epithelial cells (mTECs cell line) were maintained in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The primary tubular cells (pTECs) were prepared from renal cell suspensions as previously described. In brief, 4 weeks old Pax8-rtTAcre; MDM2f/f or MDM2f/f control mice were used for renal cell extraction. Decapsulated kidneys were diced and digested in 1 mg/ml collagenase A (Roche Diagnostics, Mannheim, Germany) for 30 min at 37 °C and then passed through 70 μm pore sieve (BD, Franklin Lakes, NJ, USA), washed, and diluted in 2 ml of PBS. Separation of the tubular segments was achieved through Percoll (31%)
Figure 4  Tubular injury in tubular cell-specific MDM2 knockout mice kidneys. (a) Representative images of PAS-stained kidney sections at a magnification 200x from Pax8rtT A-cre;MDM2f/f and control mice treated for 4, 8 or 11 days with doxycycline showing the progressive aggravation of tubular injury. At day 4 was no tubular damage apparent, at day 8 the tubular cells swelling and vacuolization were the prominent pathologic features and at day 11 the global tubular damage with massive cell loss, tubular dilation and tubular casts was detected. Tubular injury was quantified on PAS-stained renal sections as described in Materials and methods. (b) Lotus tetragonolobus lectin staining identified proximal tubuli, Tamm-Horsfall protein (THP) staining identified distal tubuli and Aquaporin 2 identified the collecting ducts in Pax8rtT A-cre;MDM2f/f and control mice kidneys treated for 4, 8 or 11 days with doxycycline. The quantitative assessment of tubuli with intact staining patterns is shown for each staining. Data are means ± S.E.M. from six mice in each group. *P<0.05, **P<0.01, ***P<0.005. All images are shown at a magnification of x100.
Figure 5 Ultrastructural pathology in tubular cell-specific MDM2 knockout mice tubular cells. (a) Electron microscopy at 4 days of MDM2 depletion revealed an onset of cytoplasmic swelling, at 8 days pronounced cytoplasmic swelling with occasional cytoplasmic membrane rupture but intact nuclei and at 11 days a complete degradation of the tubular cell structure with vacuolization of swollen cytoplasm, massive degeneration of mitochondria, edematous nuclei and release of the cell content into the tubular lumen. (EM in successive low-medium-high 1500–12 000 magnification). (b) Representative pictures of kidney sections from Pax8rtTA-cre;MDM2f/f and control mice kidneys treated for 4, 8 or 11 days with doxycycline showing staining for activated caspase-3, marker of apoptosis, Ki-67, marker of proliferation or hypertrophy and p53, marker of the cell cycle arrest, senescence and cell death. All images are shown at a magnification of ×100. Bb, brush border; BL, basal lamina; L, lysosome; M, mitochondria; N, nucleus
Figure 6 Simultaneous MDM2 and p53 tubular cell-specific knockout rescues the pathological phenotype in MDM2/p53 dKO tubulus mouse model. (a) Total mRNA was prepared from kidneys isolated from MDM2−/− tubulus/p53wt/fl or MDM2/p53dKO tubulus mice respectively and control MDM2fl/fl mice treated for 11 days with doxycyclin. The target mRNA expression levels were determined by real-time PCR and expressed as mean of the ratio versus the respective 18S mRNA level ± S.E.M. (b) Serum creatinine and BUN measurement in plasma of MDM2/p53 dKO tubulus and positive control MDM2−/− tubulus/p53wt/fl mice treated for 11 days by doxycycline (n = 10 in each group). (c) Kaplan–Meyer survival curve of MDM2/p53 dKO tubulus and control MDM2−/− tubulus/p53wt/fl mice (n = 10 in each group). (d) Representative images of PAS and p53 stained kidney sections from MDM2/p53 dKO tubulus and control MDM2−/− tubulus/p53wt/fl mice treated for 11 days with doxycycline. All images are shown at a magnification of x 200. (e) Real-time PCR analysis of renal tubular damage markers KIM-1, NGAL and TIMP-2 mRNA prepared from kidneys isolated from MDM2/p53 dKO tubulus and positive control MDM2−/− tubulus/p53wt/fl mice treated for 11 days with doxycycline. Data are means ± S.E.M. ***P < 0.005
centrifugation at 3000 r.p.m./10 min/4 °C. The pellet of tubular segments was collected and washed twice with PBS at 1500 r.p.m./5 min/4 °C. The renal tubular cell isolates were cultured under sterile conditions at 37 °C and 5% CO₂ in conditioned medium consisting of DMEM w/glucose (Gibco/Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria), HBSS (Sigma-Aldrich, Steinheim, Germany), HEPES (Gibco/Life Technologies, Grand Island, NY, USA), EGF, T3, hydrocortisone, PGE-1 and insulin transferrin sodium selenite supplement.

Figure 7  Tubular epithelium compensates only partially for the cell loss caused by MDM2 depletion. To induce a sub-lethal deletion of MDM2 in tubular epithelial cells in our mouse kidney model we treated Pax8rtTA-cre;MDM2ff/mice intermittently with 2 mg/ml doxycycline for 4 weeks. (a) Pax8rtTA-cre;MDM2ff/mice treated with intermittent regimen of doxycycline had elevated plasma creatinine and plasma BUN at 4 weeks (n = 5–6 mice in each group). (b) Representative images of kidney sections from control MDM2ff and Pax8rtTA-cre;MDM2ff/mice treated for 4 weeks intermittently with doxycycline and stained with PAS, Masson trichrome and p53 antibody. All images are shown at a magnification of x 200. (c) Real-time PCR analysis of pro-fibrotic genes mRNA prepared from kidneys isolated from experimental and control mice treated for 4 weeks with doxycycline. Data are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.005
(Roche Diagnostics, Mannheim, Germany). For the assessment of the purity of the pTECs population were the cells grown in the 8-well chamber slides to confluency.

Zebrafish experiments: Zebrafish strains and larvae were maintained as described previously. The following zebrafish strains and larvae were used:

- **mdm2 MO**: 5′-CCTCTTACCTGGCATTCTCT-3′
- **Crtl MO**: 5′-CAACCCAGGCTCTTCCTAAA-3′
- **fibronectin**: 5′-GGAATCCTCTTGATGGGGTTG-3′
- **collagen1a1**: 5′-GAATTCTTGGTAGGGGTTG-3′
- **collagen4a1**: 5′-GGAGAGCCCTTGATACCCACG-3′
- **18s**: 5′-CAACCCAGGCTCTTCCTCAA-3′

**Mice experiments.** The Pax8-rtTAcre;MDM2floxed mice were generated by breeding the MDM2floxed mice, in which loxP sites flanked the exon 4 and 5 of MDM2 gene, with Pax8-rtTAcre mice, expressing the inducible Cre recombinase under control of the Pax8 promoter. The MDM2floxed mice (B6 mixed background) were a generous gift from G. Lozano (University of Texas, Houston, USA) and the Pax8-rtTA mice (C56Bl6 background) were kindly provided by T. Huber (University of Freiburg, Freiburg, Germany). Both mice strains were previously described. 

**Renal function measurement:** Plasma creatinine levels as well as BUN (DiaSys GmbH, Holzheim, Germany) levels were determined using commercially available kits as per manufacturer’s instructions. The GFR measurement was performed by measuring transcutaneously using a USB-device according to manufacturer’s instructions (Mannheim Pharma&Diagnostics GmbH, Mannheim, Germany). The USB-device was fastened with adhesive tape to the shaved area on the mouse back. The mice were injected with FITC-Sinistrin i.v. (15 mg FITC-Sinistrin/100 g body weight). The measurement was performed for 60 min and analyzed with software provided by the manufacturer.

**Renal histology, immunohistochemistry, confocal microscopy and electron microscopy:** Kidney tissues were fixed in 4% neutral-buffered formalin, dehydrated in graded alcohols and embedded in paraffin. For routine histology the 4 μm sections were stained with periodic acid-Schiff (PAS) reagent. For immunohistochemistry sections were deparaffinized, rehydrated, transferred into citrate buffer, and either autoclaved or microwave treated for antigen retrieval and processed as described. For histochemistry we used biotinylated Lotus Tetragonolobus Lectin stain (Vector Labs, Burlingame, CA, USA). Tamm-Horsfall
protein stain (Santa Cruz, CA, USA) and rabbit anti-mouse Aquaporin 2 (Abcam, Cambridge, UK). Further on the following primary antibodies were used: rabbit anti-mouse MDM2 (Abcam, Cambridge, UK), rabbit anti-mouse p53 (Vector Laboratories, Burlingame, CA, USA), rat anti-mouse Ki-67 (DakoCytomation, Glostrup, Denmark), rabbit anti-mouse cleaved caspase-3 (Cell signaling Technology, Denver, MA, USA), mouse anti-mouse E-cadherin (BD Biosciences, San Jose, CA, USA) and mouse anti-mouse cytookeratin-7 (Abcam). Immunofluorescent stainings were evaluated using a LSM 510 confocal microscope and LSM software (Carl Zeiss AG). For transmission electron microscopy, the kidney cortex was sectioned into 1x1mm cubes and immediately immersed in fixative containing 3% glutaraldehyde and 1% paraformaldehyde in PBS. Post-fixation kidneys were immersed in cold fixative containing 2% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer (pH 7.4). Kidneys were postfixed in phosphate cacodylate-buffered 2% OsO4 for 1 h, dehydrated in graded ethanol with a final dehydration in propylene oxide and embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections (~90-nm thick) were stained with uranyl acetate and Venable’s lead citrate and viewed with a JEOL model 1200EX electron microscope (JEOL, Tokyo, Japan).

**Morphometry:** To assess tubulointerstitial changes a semiquantitative score was established to evaluate the degree and extent of tubulointerstitial damage of each field and was graded from 0 to 4 as follows: 0 represents no lesion, 1 represents tubulointerstitial damage of less than 25% per field, and 2, 3 and 4 represent tubulointerstitial damage of 25–50%, 50–75% and more than 75% of the tubulointerstitium, respectively. Approximately 30 cortical and medullary visual fields (20 x) per kidney were evaluated. Tubulointerstitial injury was defined by features such as tubular collapse, cast formation with tubular dilatation or atrophy, detachment of cells from the basement membrane.

**RNA preparation and real-time quantitative PCR.** Total RNA was extracted from mouse glomeruli, isolated using Dynabeads perfusion, or murine podocyte cell line using Ambion RNA extraction kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. After quantification, RNA quality was assessed using agarose gels before reverse transcription with Superscript II. Total RNA was prepared and real-time quantitative PCR.

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1. Shankland SJ, Wolf G. Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. Am J Physiol Renal Physiol 2000; 278: F315–F329.
2. Thomasona D, Anders HJ. Cell cycle control in the kidney. Nephrol Dial Transplant 2015; 30: 1622–1630.
3. Wittgall R. Are renal proximal tubular epithelial cells constantly prepared for an emergency? Focus on "the proliferation capacity of the renal proximal tubule involves the bulk of differentiated epithelial cells". Am J Physiol Cell Physiol 2008; 294: C1–C3.
4. Bond GL, Hu W, Levine AJ. MDM2 is a central node in the p53 pathway: 12 years and counting. Curr Cancer Drug Targets 2005; S: 3–8.
5. Thomasona D, Mulay SR, Bruns H, Anders HJ. p53-independent roles of MDM2 in NF-kappaB signaling; implications for cancer therapy, wound healing, and autoimmune diseases. Neoplasia 2012; 14: 1097–1101.
6. Voulaud KH, Lane DP. p53 in health and disease. Nat Rev Mol Cell Biol 2007; 8: 275–283.
7. Brown CJ, Lain S, Verma CS, Ferstih AR, Lane DP. Awakening guardian angels: dragging the p53 pathway. Nat Rev Cancer 2009; 9: 862–873.
8. Mulay SR, Thomasona D, Ryu M, Kulikari O, Miglonirio A, Bruns H et al. Podocyte loss involves MDM2-driven mitotic catastrophe. J Pathol 2013; 230: 322–335.
9. Mulay SR, Romoli S, Desai J, Hanasaki K, Mikhail S, Anders HJ, et al. Murine double minute-2 inhibition ameliorates established crescentic glomerulonephritis. Am J Pathol 2013; 183: 1453–1463.
10. Mulay SR, Thomasona D, Ryu M, Anders HJ. MDM2 (murine double-minute 2) links inflammation and tubular cell healing during acute kidney injury in mice. Kidney Int 2012; 81: 1199–1211.
11. Thomasona D, Bruns HA, Kretschmer V, Ebrahim H, Romoli S, Liapis H et al. Murine double minute-2 prevents p53-overactivation-related cell death (Podoptosis) of podocytes. J Am Soc Nephrol 2015; 26: 1513–1523.
12. Kobil AM, Simon O, Blumenthal A, Vogelgesang S, Dombrowski F, Amann K et al. Knockdown of ApoL1 in zebrafish larvae affects the glomerular filtration barrier and the expression of nephrotoxic nephropathy. PloS ONE 2016; 11: e0153768.
13. Bögl F, Perner B, Besenbeck B, Kothe S, Ebert C, Taudien S et al. A highly conserved retinoic acid responsive element controls wt1a expression in the zebrafish pronephros. Development 2009; 136: 2883–2892.
14. Zhang Y, Xiong S, Li Q, Hu S, Tashkori M, Van Pett C et al. Tissue-specific and age-dependent effects of global Mdm2 loss. J Pathol 2014; 233: 360–361.
15. Iwakuma T, Lozano G, MDM2, an introduction. Mol Cancer Res 2003; 1: 993–1000.
16. Ebrahim H, Mulay SR, Anders HJ, Thomasona D. MDM2 beyond cancer; podoptosis, development, inflammation, and tissue regeneration. Histol Histopathol 2015; 30: 1271–1282.
17. Allan R, Sayed SG, Kulikari O, Lichtenkert, Anders HJ. Mdm2 promotes systemic lupus erythematosus and lupus nephritis. J Am Soc Nephrol 2011; 22: 2016–2027.
18. Bohman S, Manfredi JJ. p53-independent effects of Mdm2. In: Deb P, Deb S (eds). Mutant p53 and MDM2 in Cancer. Springer: Dordrecht, Netherlands, 2014; 235–246.
19. Senturk E, Manfredi JJ. MDM2 and tumorigenesis: evolving theories and unsolved mysteries. Genes Cancer 2012: 3: 195–198.
20. Deb SP. Cell cycle regulatory functions of the human oncprotein MDM2. Mol Cancer Res 2003; 1: 1009–1016.
21. Kim ES, Shoshet JM. Reactivation of p53 via MDM2 inhibition. Cell Death Dis 2015; 6: e1395.
22. Zhao Y, Aguilar A, Bernard D, Wang S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 Inhibitors) in clinical trials for cancer treatment. J Med Chem 2015; 58: 1038–1052.
23. Monte de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995; 378: 203–206.
24. Jones SH, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995; 378: 206–208. Mdm2 is critically involved in vivo in inflammation and tubular cell healing during acute kidney injury in mice. Curr Opin Nephrol Hypertens 2015; 24: 665–670.
25. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995; 378: 203–206.
26. Hillaire S, Aboudehen K, Ye X, El-Dahr SS. Tight regulation of p53 activity by Mdm2 is required for uric acid bud branching. Dev Biol 2011; 353: 354–366.
27. Hillaire S, Ye X, El-Dahr SS. Mdm2 is required for maintenance of the nephrogenic niche. Dev Biol 2014; 397: 1–14.
28. El-Dahr S, Hillaire S, Aboudehen K, Safiudin Z. The MDM2-p53 pathway: multiple roles in kidney development. Pediatr Nephrol 2014; 29: 621–627.
29. Ringshausen I, OShea CC, Finch AJ, Swigart LB, Evan GI. Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. Cancer Cell 2006; 10: 501–514.
30. Valentine-Vega YA, Okano H, Lozano G. The intestinal epithelium compensates for p53-mediated cell death and prevents organismal survival. Cell Death Differ 2012; 17: 1722–1727.
31. Macedo E, Bouchard J, Mehta RL. Renal recovery following acute kidney injury. Curr Opin Crit Care 2008; 14: 660–665.
32. Molitoris BA, Dagher PC, Sandoval RM, Campos SB, Ashush H, Fridman E et al. siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. J Am Soc Nephrol 2009; 20: 1754–1764.
33. Wei Q, Dong G, Yang T, Megyesi J, Price PM, Dong Z. Activation and involvement of p53 in cisplatin-induced nephrotoxicity. Am J Physiol Renal Physiol 2007; 293: F476–F485.
34. Muller T, Rumpel E, Hradetzky S, Bögl F, Wegner H, Blumenthal A et al. Non-muscle myosin II A is required for the development of the zebrafish glomerulus. Kidney Int 2010; 80: 1055–1063.
35. Siegerist F, Zhou W, Endlich K, Endlich N. 4D in vivo imaging of glomerular barrier function in a zebrafish podocyte injury model. Acta Physiol 2016 (e-pub ahead of print).
36. Grier JD, Yan W, Lozano G. Conditional allele of mdm2 which encodes a p53 inhibitor. Genesis 32: 145–147.
37. Traykova-Brauch M, Schonig K, Greiner O, Miloud T, Jauch A, Bode M et al. An efficient and versatile system for acute and chronic modulation of renal tubular function in transgenic mice. Nat Med 2008; 14: 979–984.
38. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Bemá A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001; 29: 418–425.
39. Allam R, Pawar RD, Kulkarni OP, Hornung V, Hartmann G, Segerer S et al. Viral 5'-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses. Eur J Immunol 2008; 38: 3487–3496.

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