BRIEF COMMUNICATION

Murine Double Minute-2 Prevents p53-Overactivation-Related Cell Death (Podoptosis) of Podocytes

Dana Thomasova,* Hauke A. Bruns,* Victoria Kretschmer,* Martrez Ebrahim,* Simone Romoli,* Helen Liapis,† Ahmed M. Kotb,† Nicole Endlich,† and Hans-Joachim Anders*

*Nephrologisches Zentrum, Medizinische Klinik und Poliklinik IV, Klinikum der Universität, Munich, Germany; †Pathology, Immunology, and Internal Medicine (Renal), School of Medicine, Washington University, St. Louis, Missouri; and ‡Institut für Anatomie und Zellbiologie, Universitätsmedizin Greifswald, Greifswald, Germany

ABSTRACT

Murine double minute-2 (MDM2), an E3 ligase that regulates the cell cycle and inflammation, is highly expressed in podocytes. In podocyte injury, MDM2 drives podocyte loss by mitotic catastrophe, but the function of MDM2 in resting podocytes has not been explored. Here, we investigated the effects of podocyte MDM2 deletion in vitro and in vivo. In vitro, MDM2 knockdown by siRNA caused increased expression of p53 and podocyte death, which was completely rescued by co-knockdown of p53. Apoptosis, pyroptosis, pyronecrosis, necroptosis, ferroptosis, and parthanatos were excluded as modes of occurrence for this p53-overactivation-related cell death (here referred to as podoptosis). Podoptosis was associated with cytoplasmic vacuolization, endoplasmic reticulum stress, and dysregulated autophagy (previously described as paraptosis). MDM2 knockdown caused podocyte loss and proteinuria in a zebrafish model, which was consistent with the phenotype of podocyte-specific MDM2-knockout mice that also showed the aforementioned ultrastructural podocyte abnormalities before and during progressive glomerulosclerosis. The phenotype of both animal models was entirely rescued by codeletion of p53. We conclude that MDM2 maintains homeostasis and long-term survival in podocytes by preventing podoptosis, a p53-regulated form of cell death with unspecific features previously classified as paraptosis.


Podocytes are terminally differentiated epithelial cells situated at the outer surface of the glomerular capillary loops and a component of the glomerular filtration barrier (GFB). Significant podocyte loss leads to glomerular basement membrane denudation and adhesion to Bowman’s capsule with, initially, focal segmental scarring and subsequently, global glomerulosclerosis, a condition that accounts for 90% of CKD.1–4 It is rather remarkable that podocytes are able to withstand the glomerular filtration pressure as well as toxic or immunologic stress over an individual’s lifespan, but the mechanisms are not well understood. The E3 ubiquitin ligase murine double minute-2 (MDM2) regulates proteasomal degradation of the tumor suppressor gene and cell cycle regulator p53 but also, facilitates NF-κB signaling.5,6 MDM2 overexpression promotes tumor growth, whereas MDM2 antagonists are effective in cancer.7,8 We have previously shown that MDM2 drives NF-κB signaling-related inflammation in acute tubular injury, whereas its proreregenerative effect in the healing phase was exclusively p53-dependent.9 In podocyte injury, MDM2 promotes podocyte loss by bypassing cell cycle G2/M arrest and entering mitosis (i.e., mitotic catastrophe).10 However, podocytes also constitutively express high levels of MDM2,10 but what for is unknown. We speculated that resting podocytes need MDM2 to maintain homeostasis and sought to define in which manner. To test our hypothesis, we deleted MDM2 or MDM2/p53 in cultured mouse podocytes by small interfering RNA (siRNA), in zebrafish by using morpholino (MO) oligonucleotides, and by generating podocyte-specific knockout mice.

Transfection of mouse podocytes with MDM2 siRNA (Supplemental Figure 1A) resulted in prominent vacuolization (Supplemental Figure 1B) and decreased podocyte viability compared with transfection with control siRNA (Figure 1A). MDM2 suppression induced an increase of p53 mRNA and numerous p53 target genes known to regulate the cell cycle, cell death, and autophagy, such as p21, Fas, and ATG5 (Figure 1B). Simultaneous knockdown of MDM2 and p53 (Supplemental Figure 1A) completely restored podocyte viability just like the p53 inhibitor pifithrin-α (Figure 1C, Supplemental

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Correspondence: Dr. Dana Thomasova, Medizinische Klinik und Poliklinik IV der LMU, Schillerstrasse 42, D-80336 Munich, Germany. Email: dana.thomasova@med.uni-muenchen.de

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*Corresponding Author.
Figure 1. MDM2 knockdown in murine and human podocyte cell line results in decreased viability and dysregulated autophagy in podocytes. (A) Murine podocytes were incubated for 24 hours with MDM2 siRNA or control scrambled siRNA, and the cell death was measured by LDH release. Podocytes treated with 2% Triton-X were used as positive controls. (B) RNA was isolated from murine podocytes treated for 48 hours with MDM2 siRNA or control siRNA and transcribed into cDNA. Quantitative RT-PCR was performed, and mRNA
This implies that MDM2 is needed to prevent p53-overactivation-related cell death (here referred to as podoptosis). Podoptosis is thought to represent apoptosis, but pancaspase inhibition with Z-VAD FMK did not abrogate death of MDM2-depleted podocytes (Figure 1D, Supplemental Figure 2), which excludes not only apoptosis but also, caspase-1-dependent pyroptosis. Also, necrostatin-1, ferrostatin, cyclosporin A, and CA-074Me could not abrogate death of MDM2-depleted podocytes, which excludes all other known forms of regulated cell death (i.e., necroptosis, ferroptosis, parthanatos, and pyroptosis).

Because the autophagy inhibitor chloroquine rather enhanced MDM2 siRNA-induced podocyte death (Supplemental Figure 2), we further examined the distribution of markers for autophagosomes and lysosomes in a transgenic GFP-LC3 human podocyte cell line treated with MDM2 siRNA. These cells showed cytoplasmic increase of LC3-GFP punctate and redistribution and accumulation of lysosomal LAMP1 in perinuclear and perivacuolar distribution (Figure 1E), suggesting aberrations in autophagic flux. Also, p62 accumulation was apparent, indicating insufficient autophagic protein aggregate clearance as another unspecific indicator of cell stress (Figure 1E). Additionally, densitometric analysis of Western blots from MDM2 knockdown mouse podocytes incubated with chloroquine showed a slower conversion of LC3-I to LC3-II as well as accumulation of p62, indicating a low rate of autophagic flux (Supplemental Figure 1C). Furthermore, we investigated how the MDM2 deficiency in podocytes alters p53 ubiquitination and degradation. Knockdown of endogenous p53 were increased (Supplemental Figure 1D), confirming that MDM2 promotes ubiquitination and proteasome-mediated degradation of p53.

To validate the nonredundant role of MDM2 in podocytes in vivo, MDM2 was knocked down in the zebrafish model by the use of morpholinos (MOs). MOs generated against zMdm2 and a control MO were injected into fertilized eggs of the zebrafish strain ET expressing enhanced green fluorescent protein (EGFP) specifically in podocytes. The zMdm2 knockdown was confirmed by real-time PCR (RT-PCR) (Figure 2A). zMdm2 MOs treated larvae showed a strong pericardial edema compared with control MO larvae at 3 days postfertilization (Figure 2B). Histologic sections revealed the changed morphology of the glomerular tuft and the significant reduction of nephrin expression in zMdm2 knockdown larvae glomeruli compared with control MO larvae (Figure 2B). To investigate the integrity of the GFB, we used a zebrafish strain expressing enhanced green fluorescent protein (EGFP) in blood and that expresses the vitamin D-binding protein (DBP) coupled to EGFP in blood (A.M. Kotb et al., unpublished data) and does not pass the intact GFB. If the GFB becomes leaky, DBP-EGFP passes the barrier, and the fluorescence in the blood vessels decreases over time. In 4-days postfertilization-old zMdm2 MO larvae, the intensity of DBP-EGFP was significantly diminished, indicating a loss of DBP-EGFP through the filtration barrier, whereas the fluorescence in control larvae vehicles was unchanged (Figure 2C). To verify that the EGFP loss is because of damaged GFB, we injected Alexa Fluor 647-conjugated 10-kD dextran into CAE larvae and monitored its filtration into the proximal tubules. The tubular epithelial cells of control larvae showed only the dextran uptake. In contrast, zMdm2 MO larvae accumulated dextran together with DBP-EGFP, indicating disintegrated GFB (Supplemental Figure 2). Interestingly, a coinjection of MOs against zMdm2 and zp53 (1:1) to induce simultaneously zMdm2 and zp53 deficiency (Figure 2A) completely rescued the structural and functional phenotypes of the zMdm2 knockdown (Figure 2, B and C, Supplemental Figure 3). This suggests that Mdm2 is essential for podocytes to avoid p53-dependent podocyte dysfunction and loss in cultured podocytes as well as the zebrafish model.

To validate these results in the mammalian kidney, we generated mice with podocyte-specific MDM2 deletion (MDM2apodocyte) (Figure 3A). These mice were born at expected Mendelian ratios. MDM2 immunostaining was selectively diminished in podocytes of MDM2apodocyte mice compared with control littermates (Figure 3A), and MDM2 mRNA levels from purified glomeruli were also diminished (Supplemental Figure 4A). Up to 3 weeks of age, the MDM2apodocyte mice and control littermates were indistinguishable concerning weight, proteinuria, and glomerular histology. At 3–5 weeks of age, the MDM2apodocyte mice developed significant proteinuria, which increased with age (Figure 3B) and was associated with a reduced lifespan compared with control littermates up to 7 months of life (Figure 3C). MDM2apodocyte mouse kidneys were normal on light microscopy at 2–3 weeks, but at 8 weeks, collapsing FSGS was evident (Figure 3D). Glomerular lesions were associated with respective tubular atrophy and intraluminal protein casts (Figure 3D). Ultrastructural analysis showed glomerular...
Figure 2. zMdm2 knockdown in the zebrafish larvae causes malformation of the GFB and loss of nephrin. (A) After the treatment of fertilized zebrafish eggs with zMdm2 MO or zMDM2+zp53 MO, mRNA expressions of Mdm2 or both Mdm2 and p53 were significantly reduced in contrast to larvae treated with control MO. The target mRNA expression levels were determined by real-time PCR and expressed as the mean of the ratio versus the respective CTRB1 mRNA level ± SEM. Data are means ± SEMs. (B) Representative images of zebrafish larvae targeted with control MO, zMdm2 MO, and zMdm2+zp53 MO. The figure (row 1) shows a zebrafish larva (3 days postfertilization) that was treated with control MO. The glomerulus was well developed, and the podocytes (green) expressed nephrin (red) that is shown as a merged picture (yellow). In contrast, zMdm2 MO-treated larva developed a pericardial edema (arrow). The glomerular tuft is not properly formed, and the podocytes (green) have reduced nephrin expression (red). Coinjection of zMdm2 and zp53 MO (row 4) completely rescued this phenotype, and the zebrafish larva (4 days postfertilization) showed a normal developed glomerulus. The podocytes...
hyalinosis and marked abnormalities in the podocytes compared with control glomeruli. There was profound podocyte accumulation of vacuoles, enlarged lysosomes and mitochondria, endoplasmic reticulum (ER) abnormalities, and clumping of the actin cytoskeleton, suggesting significant podocyte stress (Figure 3E). At 14 weeks of age, the MDM2−/− mice displayed global glomerulosclerosis, segmental scarring, and profound podocyte vacuolization in most glomeruli and massive tubular dilation with flattened tubular epithelium and multifocal tubular casts (Figure 3D). The vacuolated podocytes stained strongly with calnexin and GRP78 at 8 weeks of age, indicating high level of ER stress (Supplemental Figure 4B). Electron microscopy identified degenerated podocytes with their cytoplasm filled with huge single membrane-bound vacuoles (Figure 3E, Supplemental Figure 5A). Podocyte counts declined with age in MDM2−/− mice, and the control group had the same count of Wilms’ tumor 1 (WT-1)/nephrin-positive podocytes per glomerulus as well as the same number of nephrons corrected to kidney planar surface (Supplemental Figure 5A). Podocyte counts declined with age in MDM2+/− mice, whereas that ratio increased in their control littermates (Figure 3E, Supplemental Figure 5B). Lack of MDM2 in podocytes was associated with an age-dependent increase of p53 positivity (Figure 3G, Supplemental Figure 5C). To investigate whether dysregulated autophagy contributes to the deleterious renal phenotype in MDM2−/− mice, we examined the kidney sections of 8- and 14-week-old mice by staining with markers of dysregulated autophagy. Confocal microscopy showed accumulation of LC3-positive autophagosomes, LAMP1- and LAMP2-positive lysosomes, and p62 protein aggregates in affected podocytes (Supplemental Figure 4C). To validate the functional role of uncontrolled p53 activity as a cause of the MDM2−/− mouse phenotype, we generated podocyte-specific double-knockout MDM2/p53Δpod mice and littermate controls with one p53 allele intact MDM2Δpod/p53+/−. Lack of p53 completely rescued the severe structural and functional phenotype of MDM2Δpod mice (Figure 4, Supplemental Figure 6).

MDM2 ubiquitinites p53 for proteasomal degradation, suppresses p53 transcription, and fosters nuclear export of p53 back to the cytoplasm.6 This way, MDM2 prevents p53-mediated cell cycle arrest, cell senescence, and premature death.8,14 For example, gain-of-function mutations in the MDM2 gene promote tumorigenesis, and specific MDM2 induction triggers lymphoma or lymphoproliferation in systemic lupus erythematosus.15,16 The disruption of MDM2 and p53 binding is being tested as a potential cancer therapy in patients who retain wild-type p53. Several MDM2 antagonists have been developed and are undergoing clinical trials.7 However, our data and the results from other studies indicate that p53 activation could have deleterious consequences in kidney, especially in podocytes and other healthy tissues. Genetic deletion of the MDM2 gene in mice results in embryonic lethality from massive p53-dependent cell death, whereas concomitant p53 deficiency completely rescues this phenotype.19,20 Specific deletion of MDM2 from the embryonic kidney compromises stem/progenitor cell renewal and differentiation.21 Our results are in line with these data and document the same nonredundant role of MDM2 in controlling p53 in terminally differentiated podocytes during kidney homeostasis. However, our results differ from those observed in other epithelial tissues. For example, MDM2 depletion in intestinal epithelial cells does not cause a persistent phenotype because of rapid clonal overgrowth of cells with insufficient Cre recombinase activity and MDM2 depletion.22 This could not occur in postmitotic podocytes that have a low turnover rate.23 Like neurons, the terminally differentiated podocytes did not retain the ability to divide, and after lost, they hardly can be replaced. Through MDM2-controlled p53 suppression, the podocytes can be driven into mitosis, which results in podocyte death by mitotic catastrophe.10 However, as our data show, the uncontrolled p53 overexpression leads to podocyte demise. Because of their complex structure, podocytes cannot be renewed by mitosis of the surviving, intact podocytes, and their permanent loss results in irreversible glomerular damage and kidney injury. This indicates that keeping p53 in balance is absolutely essential for lifelong survival of podocytes and kidney homeostasis. However, which form of podocyte death is prevented by MDM2?24 Our in vitro data exclude apoptosis, which is consistent with a previous study that excluded p53-mediated apoptotic cell death in MDM2-depleted epithelial cells.18 Our in vitro data also exclude all other known regulated cell death pathways, such as pyroptosis, pyronecrosis, necroptosis, ferroptosis, and parthanatos.11 Necroptosis was also excluded to mediate adriamycin-induced podocyte death.25 In our search for a cell death mode, we noticed dysregulated autophagy in MDM2-depleted podocytes, but this seems to be a rather unspecific phenomenon during cell stress that does not cause cell death on its own, at least not within a few weeks of life.26 Oncosis could not be formally excluded, because no inhibitors are available for this passive form of injury-related cell necrosis. However, the occurrence of podocyte death under homeostatic conditions basically excludes oncosis as an explanation for spontaneous podocyte loss. Because nowadays, cell death is no longer defined by morphologic features but by signaling pathways, the precise way of podocyte podoptosis other than apoptosis remains unknown.27 On the basis of the morphologic signature of MDM2-deficient mice,
Figure 3. Podocyte-specific MDM2 knockout in our mouse model results in p53-mediated podocyte loss and FSGS. (A) Mice expressing cre recombinase under control of podocin Nphs2 promoter were crossed with MDM2\(^{+/−}\) mice to generate podocyte-specific MDM2 knockout mice. Immunohistochemical staining confirmed MDM2 deletion in podocytes, whereas in tubular epithelial cells, the MDM2 remained unchanged. Scale bar, 20 μm. (B) MDM2\(^{−−}\)podocyte mice developed increasing proteinuria by 4 weeks of age (n=11). Light, alb/cre<5;
podocytes (massive cytoplasmic vacuolization and signs of ER stress), it may be currently classified as what has been called paraptosis-like, but the associated signaling events remain to be identified. We conclude that podocytes need MDM2 to maintain homeostasis and long-term survival. MDM2 prevents podoptosis, a p53-overactivation-related form of cell death, which in cultured podocytes, was not apoptosis or any of the known modes of regulated necrosis but showed unspecific features previously named paraptosis.

CONCISE METHODS

Cell Culture Experiments

Human podocytes expressing the GFP-LC3 transgene were provided by Tobias Huber (University of Freiburg, Freiburg, Germany). Mouse podocytes were cultured as previously described. For the viability assays, the mouse podocytes were seeded in 96-well plates (5000 cells/well). For the RNA extraction, the murine or human podocytes were cultured in six-well plates (60 000 cells/well) and kept under nonpermissive conditions (37°C in growth medium without INF-y) for 12 days to induce the differentiation of podocytes. The podocytes were then incubated with 25 nM siRNA for 24–48 hours using Lipofectamine RNAiMAX transfection reagent (Life Technologies, Darmstadt, Germany) for transient transfection. Specific siRNA to silence selectively murine and human MDM2 or murine p53 as well as appropriate control siRNA (negative control no. 1) were purchased from Ambion (Life Technologies).

At 48–72 hours post-transfection, the podocytes were collected for RNA isolation, or cytotoxicity/viability assays were performed; 16 hours after the transfection, the cells were treated with Z-VAD FMK (25 μM), necrostatin-1 (10 μM), cyclosporin A (1 μM; Enzo Life Sciences, Lörrach, Germany), chloroquine (25 μM; Invivogen, San Diego, CA), pi(3)K-alpha (10 μM; Sigma-Aldrich, Munich, Germany), ferrostatin (1 μM), and CA-074Me (500 nM; Calbiochem; EMD Millipore, Billerica, MA) for 24 hours, and viability assay was performed. The podocyte cell death and cell viability were determined using LDH assay (Cytotoxicity Detection Kit [LDH]; Roche, Mannheim, Germany) and MTT assay (Cell-Titer 96 Non-Radioative Cell Proliferation Assay; Promega, Madison, WI) according to the manufacturer’s instructions. Experiments were performed in triplicate. For immunofluorescence and confocal microscopy analysis, the human LC3-GFP podocytes were grown, differentiated (8000 cells/well) for 12 days, and transfected with 25 nM siRNA specific for human MDM2 and negative scrambled control siRNA in eight-well chamber slides coated with collagen IV (ibidi GmbH, Planegg, Germany); 48 hours after transfection, the cells were fixed in ice-cold acetone, stained using primary antibodies against LAMP-1 (Abcam, Cambridge, United Kingdom) or p62 (Progen Biotechnik, Heidelberg, Germany), and evaluated by confocal microscopy.

Zebrafish Experiments

Zebrafish were grown, mated, and maintained at 28.5°C according to standard protocols and as previously described. Embryos were kept and handled in E3 solution. Two transgene zebrafish strains were used (ET and CADE).

Imaging of the zebrafish pronephros is greatly improved in transparent zebrafish mutants. Therefore, we crossed the transparent zebrafish line Casper with the wt1aEGFP strain (provided by Christoph Englert, Jena, Germany) that expresses EGFP in podocytes. Furthermore, we crossed the transgenic l-fabp:DBP-EGFP zebrafish line (provided by Anand-Apte) expressing DBP coupled to EGFP in the blood plasma with the zebrafish strain Casper. This zebrafish line is referred to as CADE. The DBP-EGFP has the size and charge similar to albumin and cannot pass the intact filtration barrier.

Dextran Microinjection and Confocal Microscopy

Tricaine (Sigma-Aldrich) at a concentration of 0.1%–0.5% (in E3 solution) was used for anesthetizing larvae before microinjection and microscopy. The CADE larvae were injected with 20 mg/ml Alexa Fluor 647-conjugated 10-kD dextran solution (Invitrogen, San Diego, CA) into the caudal vein using Femtotips micropipettes (Eppendorf, Hamburg, Germany). After recovery in E3 medium for 40 minutes, the larvae were anesthetized again. The dextran filtration into the proximal tubules was monitored in the living zebrafish larvae by confocal microscopy 4 hours after injection. For confocal microscopy, a Leica TCS SPS (Leica Microsystems, Wetzlar, Germany) was used, and the larvae were positioned on their sides in a glass-bottomed petri dish and covered with 8% methyl cellulose in PBS. The vasculature and the pronephric tubule were imaged over time to study the distribution and the uptake of injected dextran. We used a 20X water immersion objective (HCX PL APO 20X/0.7).
Concomitant podocyte-specific MDM2 and p53 deletion rescues the podocytes in the MDM2/p53 Δpod mouse model. (A) Results from 4 months of follow-up of MDM2/p53 Δpod mice for proteinuria (8-week-old mice: n=17 control MDM2 Δpod/p53 f/wt and n=14 MDM2/Δpod, 14-week-old mice: n=10 control MDM2 Δpod/p53 f/wt and n=8 MDM2/p53 Δpod). ***P<0.005. (B) Representative images of kidney sections of 14-week-old MDM2 Δ/Δ control, MDM2 Δ/p53 f/wt, and MDM2/p53 Δ pod mice. Scale bars, 100 μm in top panel; 20 μm in middle panel. The bottom panel shows the corresponding electron micrographs in control and experimental mice, showing complete...
MO-Mediated Knockdown
MOs were designed by Gene Tools LCC (Philomath, OR). To target the 5’ untranslated region including the start codon of ZMdm2, we have used the following MO sequence: 5’-AAACACCTCCTGGGCAATTTGG-3’. As a second MO directed against a splicing site, the sequence 5’-CTCTGTTGACCATTAGTG-3’ was used. To target the 5’ untranslated region including the start codon of zp53, we have used the MO sequence 5’-GGGCTAATGCTTTGCAGAATTG-3’. For control, we have used a standard control MO provided by Gene Tools LCC with the sequence 5’-CTCTACCCATGATACATTATA-3’.

MOs were diluted to a concentration of 1 mM. A volume of approximately 3 nl per zebrafish egg was injected into the yolk at the two- to four-cell stage using a microinjector (Transjector 5246; Eppendorf).

Zebrafish Histology and Immunohistochemistry
For fluorescence microscopy of zebrafish cryosections, larvae were fixed in 2% paraformaldehyde in 1× PBS (pH 7.4) for 3 hours at room temperature and incubated in 30% sucrose at 4°C overnight. After snap freezing in liquid nitrogen using Tissue-Tek (Sakura, Staufen, Germany), cryostat sections (60 μm) were cut and permeabilized with 0.3% Triton X-100 (Merck, Darmstadt, Germany) for 30 seconds. The cryosections were stained with phalloidin (Alexa Fluor 546-conjugated phalloidin; Invitrogen) and 1 mg/100 ml Hoechst 33342 (Sigma-Aldrich) for 30 minutes. For the neprin staining, the anti-rabbit nephlin antibody (gift from A. Majumdar, Uppsala, Sweden) was incubated overnight at 4°C. After the washing steps, the Cy3-conjugated anti-rabbit secondary antibody (1:250; Dianova, Hamburg, Germany) was applied for 30 minutes.

Mouse Experiments
The MDM2fl/fl Nphs2-cre+ (MDM2p53fl/wt) mice were generated by breeding the MDM2fl/fl mice, in which loxP sites flanked exons 4 and 5 of the MDM2 gene, with Nphs2-cre+ mice, expressing the Cre recombinase under control of the podocin promoter. The MDM2fl/fl mice (B6 mixed background) were a gift from Guillermina Lozano (University of Texas, Houston, TX), and the Nphs2-cre+ mice (C56Bl/6 background) were provided by Tobias Huber. Both mice strains were previously described. The MDM2fl/fl littermates lacking the podo-cre transgene were used as control mice. To generate the podocyte-specific double-knockout MDM2fl/fl p53fl/fl mice, Nphs2-cre+ (MDM2/p53fl/fl) mice, we bred the MDM2fl/fl;Nphs2-cre+ mice with p53fl/fl mice, in which the loxP sites were inserted in introns 1 and 10 to ensure Cre-mediated removal of nearly all coding sequences. The MDM2fl/fl;Nphs2-cre+ mice were a gift from Karl Lenhard Rudolph (University of Ulm, Ulm, Germany). The MDM2fl/fl p53fl/wt;Nphs2-cre+ (MDM2p53fl/fl/p53wt) littermates were used as positive controls. All animal studies were approved by the Committee on Research Animal Care, Regierungspräsidium Oberbayern.

Renal Function Measurement
The mice urine samples were collected every week from week 2 to 17. The urine albumin was measured by the albumine ELISA kit (Bethyl Laboratories, Montgomery, TX). The creatinine kit (Diasys, Holzheim, Germany) was used for detection of urine creatinine according to the manufacturer’s instructions. Albumin-to-creatinine ratio was calculated as milligrams per deciliter albumin per milligram per deciliter creatinine.

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 reagent. For immunohistochemistry, sections were deparaffinized, rehydrated, transferred into citrate buffer, either autoclaved or microwave-treated for antigen retrieval, and processed as described. The following primary antibodies were used: guinea pig anti-mouse nephlin (1:100; Acris Antibodies, Herford, Germany), rabbit anti-mouse WT1 (1:25; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse MDM2 (1:100; Abcam, Inc.), rabbit anti-mouse p53 (1:500; Vector Laboratories, Burlingame, CA), rabbit anti-mouse LAMP1 (1:200; Abcam, Inc.), rabbit anti-mouse LAMP2 (1:250; Abcam, Inc.), guinea pig anti-mouse p62 (1:50; Progen Biotechnik), rabbit anti-mouse LC3B (1:50; Cell Signaling Technology, Danvers, MA), rabbit anti-mouse calnexin (1:100; Enzo Life Sciences, Farmingdale, NY), and rabbit anti-mouse GRP78 (1:100; Abcam, Inc.). Immunofluorescence staining was evaluated using a LSM 510 confocal microscope and LSM software (Carl Zeiss AG). For transmission electron microscopy, the kidney cortex was sectioned into 1×1-mm cubes and immediately immersed in fixative containing 3% glutaraldehyde and 1% paraformaldehyde in PBS. Postfixation 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 hour, dehydrated in graded ethanol with a final dehydration in propylene oxide, and embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections (approximately 90-nm thick) were stained with uranyl acetate and lead citrate. The sections were viewed with a JEOl model 1200EX electron microscope (JEOL, Tokyo, Japan).

RNA Preparation and Real-Time Quantitative PCR
Total RNA was extracted from mouse glomeruli and isolated using Dynabeads perfusion or the murine podocyte cell line using Ambion RNA extraction kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. After quantification, RNA quality was assessed using agarose gels before

reversal of the vacuolated phenotype in MDM2/p53p53 mice (magnifications of 4000–12,000). Scale bars, 10 μm in bottom left and bottom center; 2 μm in bottom right. (C) WT-1/nephrin costaining was used to quantify differentiated podocytes in kidneys of control MDM2p53p53/mice and MDM2/p53p53 mice kidneys. Numbers of nephrin/WT-1 double-positive cells were quantified in both groups at 3 and 14 weeks of age. The data are mean±SEM. CTRL, control; EM, electron microscopy; PAS, periodic acid–Schiff. **P<0.01 versus control group.
reverse transcription with Superscript II (Invitrogen) as described. Real-time RT-PCR was performed using SYBRGreen PCR master mix and analyzed with a Light Cycler 480 (Roche). All gene expression values were normalized using 18s RNA as a housekeeping gene. All primers used for amplification were from Metabion (Martinsried, Germany). Primers used are listed in the Table 1.

## Western Blotting and Immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Roche) and 20 mmol/L NEM (Calbiochem) followed by centrifugation at 12,000 rpm for 20 minutes.

Protein concentration was determined by Bio-Rad protein assay. For immunoprecipitation reactions, samples were incubated with 2 μg primary antibody overnight at 4°C with Dynabead-conjugated protein G (Dynal). Proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The following antibodies were used: anti-mouse MDM2, anti-rabbit β-actin, rabbit anti-mouse p53 (Abcam, Inc.), goat anti-mouse p53 (Santa Cruz Biotechnology), anti-mouse Ub (FK2; Enzo Life Sciences), rabbit anti-mouse LC3B (Cell Signaling Technology), and guinea pig anti-mouse p62 (Progen Biotechnik). The signals were visualized by an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

### Statistical Analyses

Values are expressed as means±SEMs. Statistical analyses were performed using Graphpad Prism5 software. Significance of differences was determined by the appropriate two-sided t test for single comparisons. ANOVA with post hoc Bonferroni’s correction was used for multiple comparisons. P values<0.05 were considered statistically significant.

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**DISCLOSURES**

None.

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