Tight regulation of p53 activity by Mdm2 is required for ureteric bud growth and branching

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A R T I C L E   I N F O

Article history:
Received for publication 25 October 2010
Revised 10 March 2011
Accepted 11 March 2011
Available online 21 March 2011

Keywords:
Ureteric bud
Mdm2
p53
Nephrogenesis
Branching morphogenesis
Developing kidney

A B S T R A C T

Mdm2 (Murine Double Minute-2) is required to control cellular p53 activity and protein levels. Mdm2 null embryos die of p53-mediated growth arrest and apoptosis at the peri-implantation stage. Thus, the absolute requirement for Mdm2 in organogenesis is unknown. This study examined the role of Mdm2 in kidney development, an organ which develops via epithelial–mesenchymal interactions and branching morphogenesis. Mdm2 mRNA and protein are expressed in the ureteric bud (UB) epithelium and metanephric mesenchyme (MM) lineages. We report here the results of conditional deletion of Mdm2 from the UB epithelium. UBmdm2−/− mice die soon after birth and uniformly display severe renal hypoplasia due to defective UB branching and underdeveloped nephrogenic zone. Ex vivo cultured UBmdm2−/− explants exhibit arrested development of the UB and its branches and consequently develop few nephron progenitors. UBmdm2−/− cells have reduced proliferation rate and enhanced apoptosis. Although markedly reduced in number, the UB tips of UBmdm2−/− metanephroi continue to express c-ret and Wnt11; however, there was a notable reduction in Wnt9b, Lhx-1 and Pax-2 expression levels. We further show that the UBmdm2−/− mutant phenotype is mediated by aberrant p53 activity because it is rescued by UB-specific deletion of the p53 gene. These results demonstrate a critical and cell autonomous role for Mdm2 in the UB lineage. Mdm2-mediated inhibition of p53 activity is a prerequisite for renal organogenesis.

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Introduction

Congenital abnormalities of the kidney and urinary tract account for 30–40% of end-stage renal disease in infants and children (Bates, 2000; Cain et al., 2010). The etiology of a majority of them arises from defects in the fine balance between proliferation, survival, differentiation and the acquisition of physiological function. The definitive kidney or metanephros develops through reciprocal signaling between the ureteric bud (UB), an epithelial outgrowth of the Wolffian or mesonephric duct, and the metanephric mesenchyme (MM) (Costantini and Kopan, 2010; Lechner and Dressler, 1997). These proximate interactions result in reiterated branching of the UB leading to the formation of an elaborate collecting duct system. Meanwhile the MM assumes one of three fates, namely, epithelial renal vesicles (which will ultimately form the mature nephrons), stroma, or vascular elements (Dressler, 2009). Disruptions, either genetic or chemical, of the reciprocal signaling pathways result in impaired growth and/or differentiation of the component tissues leading to agenesis or dysgenesis of the kidney (Cain et al., 2010).

Tightly coordinated cell death and survival decisions have a direct impact on embryonic development and in adult tissue homeostasis.

Deregulation of these processes results in pathological conditions. Owing to the growth inhibitory and apoptosis-promoting functions of the tumor suppressor p53, there are strict mechanisms in place to regulate p53 levels and activity under normal physiological conditions (Harris and Levine, 2005; Oren et al., 2002; Poyurovsky and Prives, 2006). Mdm2 is recognized as a potent negative regulator of p53 stability and function (Lozano and Montes de Oca Luna, 1998; Wade et al., 2010). Mdm2 was originally identified as an amplified gene on double-minute chromosomes in transformed mouse fibroblasts (Fakharzadeh et al., 1991). Subsequently, it was found to interact with p53 and to inhibit its function through two main mechanisms: first, the direct binding of Mdm2 to the N-terminal domain of p53 inhibits the transcriptional activation function of p53 (Momand et al., 1992; Ollner et al., 1993); second, Mdm2 possesses E3 ubiquitin activity that targets p53 for degradation through the 26S proteasome (Haupt et al., 1997; Kubb utat et al., 1997). The vital interaction between Mdm2 and p53 clearly revealed by elegant genetic studies which showed that the peri-implantation lethality of Mdm2 null mice can be circumvented by the concomitant deletion of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). Remarkably, Mdm2 itself is in turn a transcriptional target of p53 and together they form an autoregulatory feedback loop (Barak et al., 1993; Perry et al., 1993). In contrast, the closely related partner, Mdm4, is not transcriptionally regulated by p53 (Marine et al., 2007).

The Mdm2−/−p53 signaling network has been implicated in the development and/or homeostasis of several cell lineages. Genetic
mouse models that are homozygous null, hypomorphic or haploinsufficient for Mdm2 demonstrate that the Mdm2–p53 signaling network supports the timely proliferation, differentiation, and survival of several cell lineages namely hematopoietic, lymphopoietic, cardiomyocyte, osteoblast, hepatocyte, neural, and intestinal cells (Francoz et al., 2006; Grier et al., 2006; Lengner et al., 2006; Mendrysa et al., 2003; Terzian et al., 2007; Xiong et al., 2007). Notably, most (but not all) phenotypes associated with either a partial or total loss of Mdm2 from selected tissues are efficiently rescued by the concomitant deletion of p53 or a reduction in p53dosage. In certain tissues (e.g., heart), other members of the Mdm2 family, such as Mdm4, exert redundant developmental functions with Mdm2. With regard to the kidney, there is suggestive evidence that Mdm2 or Mdm4 gene function is necessary for normal kidney development. Mdm2+/−; Mdm4+/- double heterozygous mice that survived to postnatal day (P) 20 have small kidneys with very few glomeruli in addition to marked hypoplasia and/or atrophy of the thymus and spleen (Terzian et al., 2007). Using transgenic mice that express wild type p53 within the ureteric bud, Godley et al. (1996) demonstrated the need to titrate the expression levels of p53 for kidney development to proceed normally. These transgenic mice exhibit acute kidney degeneration at E17.5 reaching half the size of normal kidneys with fewer glomeruli that are hypertrophic and fibrotic. Excessive p53 interferes with the differentiation of the ureteric buds and secondarily causes apoptosis in the adjacent metanephric mesenchyme and its limited conversion to epithelium (Godley et al., 1996). Given that Mdm2 is a potent negative regulator of p53, by extrapolation it may be inferred that Mdm2 could have a significant role in embryonic kidney morphogenesis. Here, we have characterized the spatial and temporal expression of Mdm2 in the developing kidney in mice. Utilizing a conditional approach, we examined the consequences of Mdm2 loss exclusively from the UB lineage on metanephric development. We demonstrate that Mdm2 has an essential role in maintaining normal branching morphogenesis and/or survival of the UB epithelium. Strikingly, the loss of Mdm2 appears to disrupt Wnt9b, Lhx-1 and Pax2 signaling, essential for the reciprocal induction of metanephric mesenchyme and the organization of epithelial progenitors of renal vesicles. Genetic rescue experiments suggest that the renal phenotypes encountered were largely p53-dependent.

Materials and methods

Animals

All animal protocols utilized were in strict adherence to guidelines established by the Institutional Animal Care and Use Committee. For timed pregnancies, noon of the day on which the vaginal plug was detected was regarded as embryonic day (E) 0.5. The Mdm2-fllox mice (01XH9, Dr. Mary Ellen Perry) were obtained from the NCI mouse repository (Frederick, MD) while the R26R-EYFP reporter mice (006148, Dr. Frank Constantini) were purchased from the Jackson Laboratory (Maine, USA). The specifications for genotyping were furnished by the respective companies. The Hoxb7-Eyg–Cre mice (Zhao et al., 2004) were a kind gift from Dr. Carlton Bates. The breeding strategy used involved crossing Hoxb7–Eyg–Cre; mdm2fllox/+; transgenic mice to either mdm2fllox/fllox or R26R.EYFP+; mdm2fllox/+ mice. For our rescue experiments we used both conventional and conditional approaches: accordingly we bred (1) Hoxb7–Cre+; p53+/−; mdm2fllox/+ mice to Hoxb7–Cre+; p53+/−; mdm2fllox/+ mice or (2) Hoxb7–Cre+; p53fllox/+; mdm2fllox/+ mice to Hoxb7–Cre+; p53fllox/+; mdm2fllox/+ mice.

Reverse transcriptase-PCR

RNA was isolated from the kidneys using the Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The RNA was reverse transcribed using Random Hexamers and a first-strand synthesis kit (SuperScript II; Invitrogen) according the manufacturer’s recommendations. The forward and reverse primers for Mdm2 had the following sequence: 5’ ATG TGC AAT ACC AAC ATC TCT GTG TC 3‘ and 5’ GCT GAC TTA CAG CCA CTA AAT TTC 3‘ respectively and yielded a 337 bp PCR product. The PCR product corresponded to nucleotides 202–538 of the mouse Mdm2 sequence deposited under GenBank Accession number X58876. The cycling parameters used were 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min.

Quantitative reverse transcriptase–PCR

Quantitative reverse transcriptase–PCR was performed on total RNA isolated from embryonic kidneys using the RNeasy Mini Kit (Qiagen). Real-time primer-probe mixes for Mdm2 (ID Mm00487652-g1 Mdm2), p53 (ID Mm01731287_m1), and Gapdh (Mm99999915-g1) were ordered from Applied Biosystems. The Taqman expression assay was constituted using reagents from the Brilliant II QRT–PCR 1-step Master mix kit (Cat #600809, Agilent Technologies). The thermal profile used was as follows: 50 °C for 30 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 56 °C for 1 min and 72 °C for 30 s. The reactions were done in triplicate. The scale bars represent the standard error of mean.

Gross morphology

All bright field images were captured on a Nikon SMZ1000 stereomicroscope mounted with a DS–Fi1 camera with the aid of NIS-Elements F2.20 software.

Histological staining

Periodic acid-Schiff staining (PAS)

PAS staining on paraffin tissue sections was done using the Periodic Acid–Schiff Stain Kit (Richard–Allan Scientific). Briefly, deparaffinized and rehydrated sections were incubated in Periodic acid solution for 5 min and Schiff Reagent for 15 min. Hematoxylin 1 was used to stain the nuclei and Bluing Reagent to intensify the color. The slides were finally dehydrated in graded alcohol series, cleared in Xylene and mounted using Permount mounting media.

Hematoxylin and Eosin staining

Routine histological staining using H and E involved incubation in Hematoxylin 2 for 2 min and Eosin Y (Richard–Allan Scientific) for 1 min.

Organ culture

Ex vivo kidney explants were grown in 6-well transwell plates with 0.4 μm pore size filters (Corning Inc). E12.5 kidneys were cultured in Advanced DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin streptomycin at the air and medium interface in 5% CO2 atmosphere at 37 °C. To study branching morphogenesis the kidney explants were cultured for 48 h and images captured using an Olympus BX51 fluorescence microscope.

Immunohistochemistry

The kidneys were fixed in 10% buffered formalin and processed for paraffin embedding. Four micrometer paraffin sections were subjected to antigen retrieval (10 mM sodium citrate, pH 6.0) after deparaffinization and rehydration steps. Primary antibodies used were polyclonal rabbit anti-Mdm2 at 1:100 dilution (ab15471-1 Abcam), Dolichos biflorus lectin (1:40 dilution, Sigma) and rabbit anti-Pax2 at 1:100 dilution (Zymed), Fluorescein Lotus Tetragonolobus lectin at 1:100 dilution (FL–1321, Vector Laboratories, Inc.), polyclonal goat anti-AQP2 at 1:500 dilution(sc-9882, Santa cruz), rabbit anti-
cleaved caspase 3 (Asp 175) at 1:200 dilution (9661, Cell Signaling),
rabbit phosphor-histone H3 (Ser 10) antibody at 1:100 dilution (9701,
Cell Signaling), monoclonal mouse anti-pan cytokeratin at 1:100
dilution (Sigma), polyclonal rabbit anti-WT1 (C-19:sc-192, Santa
Cruz) at 1:200 dilution, monoclonal mouse anti-E-cadherin (1:100
dilution; 610181, BD Biosciences), polyclonal rabbit anti-K-cadherin
(1:100 dilution; cadherin-6, gift from G. Dressler), polyclonal rabbit
anti-Six2 (1:200 dilution; 11562-1-AP, Proteintech), and DAPI (1:500
dilution D1306, Invitrogen). The peroxidase based Vectastain ABC
elite kit (Vector Laboratories, Inc) was used for DAB detection. For
immuno-fluorescence detection we used donkey anti-mouse or
donkey anti-rabbit secondary IgG antibodies with Alexa
fluor 555 or 488 conjugates (Invitrogen). The immunofluorescent images were
captured using a 3D or deconvolution scope (Leica DMRXA2).

**TUNEL assay**

Recombinant Terminal Deoxynucleotidyl transferase (rTdT) me-
diated nick-end labeling (TUNEL) was performed using the Dead End
Fluorometric Tunel System (Promega) according to the manufac-
turer's guidelines. Four micrometer paraffin sections were fixed in
methanol-free paraformaldehyde before and after proteinase K
treatment at 20 μg/ml for 8–10 min at room temperature. The sections
were incubated with the nucleotide mixture (which included
fluorescein-tagged dUTP) and rTdT enzyme for an hour at 37 °C. The
slides were mounted using Vectashield with DAPI (Vector Laborato-
ries, Inc). The images were captured using a deconvolution fluores-
cent scope. Quantitative analysis of apoptotic foci to area ratio was
determined using Slidebook 4.1 (Digital Microscopy Software).

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**Fig. 1.** Expression of Mdm2 and p53 during kidney development in the mouse. (A) Semi-quantitative and (B) real-time quantitative RT-PCR of p53 and Mdm2 mRNA. Total RNA was extracted from kidneys of representative embryonic (E) stages, newborn (NB), postnatal (P) and adult mice. Spatial distribution of Mdm2 (Panels E, G, I, and K) and p53 (Panels F, H, J, and L) transcripts during early metanephrogenesis. Panels C and D represent the sense controls for Mdm2 and p53 at E11.5. Mdm2 and p53 transcripts are present in the nephric duct, mesonephric tubules and in the developing metanephros at E11.5 (Panels E, F). Examination of the metanephros from E11.5–13.5 revealed that both the UB and MM are enriched for
p53 and Mdm2 transcripts (Panels E–L). (M, N) Immunostaining of Mdm2 in E14.5 kidney. The ureteric bud lineage, the precursors of the nephron, the glomeruli, and the stroma are
Mdm2-positive. UB: ureteric bud; SB: S-shaped body; nd: nephric duct.
Whole mount in situ hybridization

The protocol used was established in the De Robertis laboratory and can be accessed under mouse protocols (http://www.hhmi.ucla.edu/derobertis). The non-radioactive RNA probes carried a digoxigenin label which was detected using anti-digoxigenin Fab fragments coupled to alkaline phosphatase (Roche Diagnostics). BM purple was used as the chromogenic substrate for Alkaline Phosphatase. The Mdm2 probe was generated by in vitro transcription from a pCMV-SPORT6 expression vector carrying full length Mdm2 corresponding to Accession no BC092270 (Clone ID4018419 Mouse MGC Verified FL cDNA; Open Biosystems). We linearized and reverse transcribed with EcoRV/T7 for antisense probe and HindIII/SP6 for sense probe. The probe length was approximately 1.4 kb. Other probes used in our assays were Pax2, Utx1, cRet, Wnt11, Wnt9b, and p53.

Results

Spatio-temporal expression of Mdm2 and p53 in the developing kidney

Initial studies of Mdm2 in the mouse reported low level ubiquitous expression throughout the embryo and in the adult tissues analyzed (Montes de Oca Luna et al., 1995). Here, we examined Mdm2 and p53 gene expression in mouse embryonic (E) and postnatal (P) kidneys. RT-PCR (Fig. 1A) and real-time quantitative PCR (Fig. 1B) showed that expression of both genes follows a similar temporal profile, being high in the embryonic kidney followed by a gradual decline in gene expression postnatally. In situ hybridization revealed that Mdm2 and p53 mRNAs are expressed in the epithelial and mesenchymal compartments of the developing kidney (Figs. 1C–L). From E11.5 to E13.5, both transcripts are expressed in the Wolffian duct, the mesonephric tubules, the UB, and the MM. Immunostaining of E14.5 kidney sections revealed widespread expression of Mdm2 in the UB epithelium, the nephron progenitors, and the cortical and medullary stroma (Figs. 1M, N). This spatio-temporal expression pattern suggests a significant role for the Mdm2–p53 pathway during nephrogenesis.

Deletion of Mdm2 from the UB lineage disrupts metanephric development

To determine if Mdm2 is required for branching morphogenesis in the developing metanephros, we crossed mice harboring Mdm2 floxed alleles (Mdm2F/F) (Mendrysa et al., 2003) (Suppl. Fig. 1A) to a Hoxb7-Cre transgenic line that drives Cre recombinase expression specifically in the UB lineage (Zhao et al., 2004). The progeny are hereafter designated as wild type (UB^{mdm2+/+}), heterozygous null (UB^{mdm2+/−}) or homozygous null (UB^{mdm2−/−}). To confirm the specificity of Cre recombinase activity in our system, we crossed Mdm2F/+; Hoxb7-Cre^{ERT2/}+ mice with R26REYFP/+; Mdm2F/+ littermates. The EYFP reporter gene is transcriptionally silent until Cre-mediated recombination removes the floxed stop cassette upstream from its coding sequence. At E11.5, we find EYFP reporter expression in the nephric duct, the mesonephric tubules, and the UB. Conversely, the R26R^{ERT2/}; Mdm2^{F+} littermate but without the Hoxb7-Cre transgene does not

Fig. 2. Ureteric bud-specific deletion of Mdm2 results in bilaterally small, hypoplastic kidneys. Gross morphology of P1 kidneys isolated from wild type (A) and conditional homozygous null mutant (B) littermates. Note; the contralateral adrenal gland in Panel B was lost during dissection. (C–H) Histological staining of kidney sections at P1 reveals severe structural anomalies in UB^{mdm2−/−} mice. The mutant kidneys have poor tissue organization and numerous dilated tubules resembling cysts occupy the bulk of the kidney. The cortical and medullary zones are not identifiable. C, D, F, and G represent Hematoxylin and Eosin staining while E and H represent Periodic acid Schiff staining. Ub: ureteric bud; RV: renal vesicle; PT: proximal tubule, NZ: nephrogenic zone.
show EYFP expression (Suppl. Fig. 1B). In situ hybridization at E13.5 confirmed a marked reduction of Mdm2 transcripts in UB<sup>mdm2−/−</sup> kidneys (Suppl. Fig. 1C).

At P1, UB<sup>mdm2−/−</sup> kidneys were remarkably smaller and hypodysplastic compared to those of UB<sup>mdm2+/−</sup> and UB<sup>mdm2+/+</sup> littermate controls (Figs. 2A, B). Histological analysis of the control and null mutant kidney sections at P1 showed the presence of numerous cyst-like dilated tubules that occupied the bulk of the null mutant kidney. Notably, UB<sup>mdm2−/−</sup> kidneys lacked normal hist-architecture with no demarcation of cortical and medullary zones (Figs. 2C–H).

Analyses of P1 kidney sections by immunohistochemistry was undertaken to reveal the tissue compartments most affected by the elimination of Mdm2. There were only a few <i>Dolichos biflorus</i> lectin-staining collecting ducts in the UB<sup>mdm2−/−</sup> kidney sections relative to the control (Suppl. Figs. 2A, B). This was indicative of severely impaired branching morphogenesis of the UB. In these mutants, UB branches failed to extend to the periphery of the kidney. Antibodies to Pax-2 revealed that the nephron progenitors were ill developed in conjunction to the poorly developed UB lineage (Suppl. Figs. 2C, D). Clearly, the nephrogenic zone, which houses actively proliferating UB tips, nephron progenitors, and cortical stroma, was poorly developed in the mutant UB<sup>mdm2−/−</sup> kidneys. As expected, proximal tubulogenesis (LTA-positive tubules) was less affected than UB branching (Suppl. Figs. 2E, F). In contrast, AQP-2 water channel, a marker of the principal cells of the collecting ducts, was expressed in few collecting ducts (Suppl. Figs. 2G–J). Therefore, renal development was compromised in mutant UB<sup>mdm2−/−</sup> secondary to UB growth and branching defects.

**Requirement for Mdm2 in early UB branching morphogenesis**

Having established that Mdm2 is necessary for kidney development, we wanted to determine the developmental stage at which the defect becomes first evident. In the conditional UB<sup>mdm2−/−</sup> mutant kidneys,

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**Fig. 3.** Loss of Mdm2 results in an early impairment of UB branching morphogenesis. Wild type (A–C) and UB<sup>mdm2−/−</sup> (D–I) explants were harvested at E12.5 and cultured for 48 h; UB branching was visualized by EYFP fluorescence. The UB bifurcates only a couple of times before it arrests (Panels E, F), and in some cases, the UB invades the mesenchyme but fails to bifurcate (Panels H, I). Kidneys from control and UB<sup>mdm2−/−</sup> littermates collected at E12.5 (Panels J and K) and E14.5 (Panels L and M) and stained with anti-pan cytokeratin to reveal the extent of UB branching. The UB makes it to the T-stage in the UB<sup>mdm2−/−</sup> kidneys but arrests shortly thereafter.
the UB invades the MM on time and progresses to the T-stage at E11.5 as in controls, although the first branching pattern appears dysmorphic and occasionally fails to occur altogether (see subsequent discussion). We followed branching morphogenesis of E12.5 UB\textsuperscript{mdm2−/−} and littermate control kidney explants ex vivo. During 24–48 h in culture, the explants of UB\textsuperscript{mdm2−/−} grow very poorly and the UB either branches once or twice or fails to branch as compared to the wild type UB which undergoes remarkable growth and branching (Figs. 3A–I). The divergence in branching pattern was most apparent at E12.5 with a significant drop in the number of UB tips and branch points and consequently visibly smaller kidneys during later organogenesis. Branching of the UB appears to arrest following the first two generations of branching, although elongation remains less affected (Figs. 3J–M).

Immunostaining of sections from E14.5 and E15.5 explants with antibodies to Pax2 (a marker of epithelial nephron progenitors) and cytokeratin (a marker of the UB branches) revealed a marked diminution of UB branches and tips (Figs. 4A–D, A′, B′; G, H, G′, H′; the cap mesenchyme (CM) is present but highly disorganized in UB\textsuperscript{mdm2−/−} as compared to wild type kidneys (Figs. 4E, F). By E15.5, wild type UB branches have elongated and reached the periphery of the cortex; in contrast, few if any UB\textsuperscript{mdm2−/−} stalks or tips are observed in the nephrogenic zone (Figs. 4G, H). The loss of UBs was accompanied by a secondary reduction in nascent nephrons (e.g. renal vesicles, RV) (Figs. 4L, J). A more extensive analysis of the progenitor cell population at E14.5 using Six2 antibodies confirms that Six2+ CM cells are present in the dysplastic UB\textsuperscript{mdm2−/−} kidneys and surround the remnant UB branches; however, the cap mesenchyme is clearly disorganized (Figs. 5A–F and Suppl. Fig. 3). As a result, fewer nephrons are formed in UB\textsuperscript{mdm2−/−} kidneys. This is illustrated by the paucity of WT1+ glomeruli in mutant as compared to wild type kidneys (Figs. 5G–L and Suppl. Fig. 3).

Loss of Mdm2 from the UB induces apoptosis and growth arrest

In order to understand the cellular mechanism(s) leading to impaired UB branching in the UB\textsuperscript{mdm2−/−} mutants, we assayed for cell apoptosis and cell proliferation. We used the TUNEL assay to examine cell apoptosis at E14.5. Given that the mutant kidneys were significantly smaller in size relative to the controls we considered the number of apoptotic foci to area ratio. The UB epithelium was labeled by antibodies to cytokeratin. Subsequent to the loss of Mdm2 from the UB, we find that apoptosis is induced in the UB branches of UB\textsuperscript{mdm2−/−} kidneys (compare panels A′ vs. B′; G′ vs. H′). The cap mesenchyme (CM) appears intact but disorganized (compare E vs. F).
UB<sup>mdm2−/−</sup> mutants at E14.5 (Figs. 6A–D). TUNEL+ cells were also found in the ureter of UB<sup>mdm2−/−</sup> kidney (Figs. 6E–H). At E16.5, we observed widespread and extensive apoptosis in both the UB and MM (data not shown). Quantitative analysis demonstrated that the rate of apoptosis is approximately 3.0-fold higher in the UB<sup>mdm2−/−</sup> than control kidneys (Fig. 6I). Immunostaining with anti-cleaved caspase-3 antibodies, another indicator of apoptosis, confirmed marked apoptosis in the UB branches and ureter of UB<sup>mdm2−/−</sup> kidneys (Figs. 7A–T). Notably, the cleaved caspase-3 staining in the conditional mutants has an unusual sub-cellular localization, being largely restricted to the apical borders of the UB cells. In all probability, this marks a very early apoptotic event in these cells since the nuclear morphology/density in the activated caspase-3 staining cell population is normal. These cleaved caspase-3 positive bodies could be related to mitochondria or some kind of early apoptotic vesicles. In conjunction with caspase-mediated apoptosis, the UB<sup>mdm2−/−</sup> kidneys also showed fewer phospho-histone3 staining foci per area relative to those of littermate controls (Figs. 8A–F). Quantitative analysis confirmed that reduced cell proliferation occurs in the homozygous UB<sup>mdm2−/−</sup> but not in heterozygous littermates (Fig. 8C). Thus, decreased cell viability and cell proliferation could explain the hypoplastic kidneys in the mutant mice.

Deletion of p53 from the UB lineage rescues renal development in UB<sup>mdm2−/−</sup> mice

Mdm2 elimination leads to unchecked p53 activity with lethal consequences (de Rozieres et al., 2000). In these instances, the concomitant loss of p53 was enough to restore viability. Therefore, we asked if Mdm2 regulation of p53 is vital to UB branching morphogenesis and nephrogenesis. To this end, we used a genetic rescue approach that involved crossing Hoxb7<sup>−/−</sup>: Mdm2<sup>+/−</sup> mice to p53<sup>−/−</sup> mice (Jacks et al., 1994) or p53<sup>−/−</sup> mice (Saifudeen et al., 2009). The results revealed that deletion of one p53 allele on UB<sup>mdm2−/−</sup> background (UB<sup>mdm2−/−; p53<sup>−/−</sup>−/−</sup>) was not sufficient to mount a full rescue of the renal phenotype. Significantly, elimination of both p53 alleles from the whole kidney anlagen (UB<sup>mdm2−/−; p53<sup>−/−</sup>−/−</sup>−/−) or specifically from the UB lineage (UB<sup>mdm2−/−; p53<sup>−/−</sup>−/−</sup>) rescues postnatal survival (Tables 1 and 2). The kidney size and histo-architecture of the rescued mice at birth are comparable to those of control wild type mice from the same litter (Figs. 9A–C). Moreover, the rescued embryos exhibit normal branching of the UB (Figs. 9D–I).

Ureteric bud specific loss of Mdm2 disrupts gene expression

To examine the impact of Mdm2 deletion on some of the molecular mediators of UB branching morphogenesis, we analyzed their expression in the null mutants by in situ hybridization. At E11.5, c-Ret, a receptor tyrosine kinase for GDNF, is expressed in the WD, UB and its branches (Fig. 10A). The expression of c-Ret was maintained in the UB<sup>mdm2−/−</sup> mutants although the first branching usually resulted in mis-shaped T (Fig. 10B). Notably, Wnt9b is suppressed in the WD and UB at E11.5 (Figs. 10C, D). Release of Wnt9b from the UB initiates the nephrogenesis program by activating β-catenin signaling and mesenchyme-to-epithelium transition. At E12.5, downregulation of Wnt9b in the UB branches of UB<sup>mdm2−/−</sup> became more evident but this
may simply reflect reduced cell numbers at this point (Figs. 11A, B). The reduced number of UB tips in the UBmdm2–/– correlated with fewer Wnt11 expressing-UB tips at E12.5 (Figs. 11C, D). Notably, Lhx1 and Pax2 were markedly decreased reflecting reduced number of induced nascent nephrons in UBmdm2–/– kidney showing excessive apoptosis in the mutant ureteric epithelium. (I) Quantitative analysis of the number of apoptotic foci per field area. There is a 3-fold elevation in the levels of apoptosis in the UBmdm2–/– compared to wild type and heterozygous mutant kidneys.

### Discussion

The present study demonstrates that kidney-specific inactivation of Mdm2 results in renal hypodysplasia that is mediated by p53-mediated growth arrest and apoptosis. Thus, tight regulation of p53 by Mdm2 is absolutely required to sustain branching morphogenesis in the developing kidney.

Control of p53 activity by Mdm2 is indispensable for embryogenesis and organogenesis to progress normally. Pursuant to the loss of Mdm2 there is an increase in the levels and/or activity of p53 compromising viability of the embryo (Chavez-Reyes et al., 2003; de Rozieres et al., 2000; Montes de Oca Luna et al., 1995). Our study extends these global embryonic functions of Mdm2 demonstrating an absolute requirement for Mdm2 in mammalian renal organogenesis. This conclusion is based on the following evidence. The temporal and spatial expression of p53 and its transcriptional target, Mdm2, in the UB and surrounding MM is consistent with a role of the Mdm2-p53 pathway in nephrogenesis. UB-specific elimination of Mdm2 results in bilaterally small hypodysplastic kidneys with few collecting ducts and isolated glomeruli. Consequently, UBmdm2–/– mice die shortly after birth and do not survive to weaning. An early arrest of UB branching morphogenesis detected both in vivo and in ex vivo cultures of UBmdm2–/– kidneys associated with extensive apoptosis in the UB epithelium and reduced cell proliferation. Furthermore, targeted inactivation of Mdm2 in the UB results in downregulation of key developmental regulators, e.g., Wnt9b at E11.5 and Lhx1 and Pax2 at E12.5–13.5. When the loss of Mdm2 in the UB was combined with the loss of p53 either in the UB lineage or in the germline, we observed a complete rescue of the renal phenotype, which in turn allowed the survival of the compound mutant mice to adulthood. Interestingly, even 50% gene dosage of p53 proved detrimental to proper UB morphogenesis in the absence of functional Mdm2. Thus, the tight regulation of p53 function by Mdm2 is a prerequisite for mammalian nephrogenesis.

The reiterative pattern by which the UB epithelia arborize is governed by factors that promote its proliferation and migration and those that repress ectopic budding and improper branching (Bush et al., 2006; Costantini, 2006; Costantini and Koman, 2010). Our study demonstrates that Mdm2 regulation of p53 stability/activity is necessary to sustain branching morphogenesis and to ensure proper patterning as it dichotomizes during nephrogenesis. The importance of the Mdm2–p53 nexus to renal organogenesis is underscored by the complete rescue of branching morphogenesis, kidney size, and cytoarchitecture upon concomitant p53 loss in our genetic models. Although the present study was not designed to investigate the precise signaling pathways (downstream of p53) which negatively affected renal development in the absence of Mdm2, it is likely that the molecular pathogenesis of the renal defect is complex and multifactorial considering the pleiotropic role of p53 in cellular homeostasis. For example, it was reported that the PI3K/PTEN node is essential for the proper patterning of the dichotomizing UB in response to GDNF-c-Ret signaling (Kim and Dressler, 2007). According to this model, PTEN antagonizes PI3K function allowing the accumulation of inositol triphosphates only at the leading edge of the growing UB epithelium. This allows directed cell migration in response to GDNF in the metanephric mesenchyme. Akt and Mdm2 are downstream effectors of the PI3K pathway that ensure cell survival, growth, proliferation and migration (Mayo and Donner, 2002). On the other hand, PTEN is a transcriptional target of p53 and a mediator of p533-triggered apoptosis (Stambolic et al., 2001). It is tempting to speculate that the targeted inactivation of Mdm2 in the UB lineage might be altering the fine balance exerted by the PI3K/PTEN node essentially compromising branching morphogenesis.

Mdm2 can be envisioned as forming the hub through which multiple mitogenic signals are coordinated. During embryogenesis, HGF–Met signaling through PI3K–Akt–mTOR triggers Mdm2 upregulation which ensures survival of the hepatocytes (Moumen et al., 2007). Ishibe et al. (2009) demonstrated that mice with genetic disruption of Met and Egrf signaling in the UB cells show a marked decrease in UB branching leading to small kidneys, renal failure and lethality. This phenotype is reminiscent of UBmdm2–/– mice.

The constitutive regulation of p53 by Mdm2 is essential for organ development and tissue homeostasis to proceed normally. Thus, a third mechanism by which loss or downregulation of Mdm2 affects kidney development is via unchecked p53-mediated apoptosis or cell cycle arrest, as demonstrated in studies of other organs, such as the lymphocytes, osteoblasts, neuronal progenitors/postmitotic neurons, cardiomyocytes, smooth muscle cells of the GI tract, and epithelial...
cells (Boesten et al., 2006; Francoz et al., 2006; Grier et al., 2006; Lengner et al., 2006; Mendrysa et al., 2003; Xiong et al., 2006). As is the case with our study, rescue of the mutant phenotype was achieved only by the complete elimination of p53. Our attempts to show accumulation of the p53 protein in UB<sup>Mdm2<sup>−/−</sup></sup> kidneys were hampered by the lack of reliable p53 antibodies that recognize mouse p53 on tissue sections. Regardless, the complete rescue of the renal Mdm2-null phenotype by germline or lineage-specific p53 deletion provides a direct genetic proof that the observed phenotype is p53-mediated.

Fig. 7. Activation of caspase-3 mediated apoptosis in UB<sup>Mdm2<sup>−/−</sup></sup> kidneys. Double Immunostaining for cytokeratin (green) and activated caspase-3 (red) in sections of E14.5 control (Panels A, C-J, S) and mutant UB<sup>Mdm2<sup>−/−</sup></sup> (Panels B, K-R, T) kidneys demonstrates that the effector of apoptosis, caspase 3, is induced in mutant UB<sup>Mdm2<sup>−/−</sup></sup> cells. Nuclei are counterstained with DAPI (blue).
A hypomorphic mouse model of Mdm2 shows that Mdm2 function extends beyond its housekeeping roles to encompass developmental processes and organogenesis (Mendrysa et al., 2003). These studies established that constitutive regulation of p53 by Mdm2 was necessary in homeostatic tissues of postnatal mice. Significantly, these mice presented with reduced body weight, lymphopenia and with severe defects in hematopoiesis, all phenotypes being p53 dependent (Mendrysa et al., 2003). Lengner et al. (2006) followed the effects of complete loss of Mdm2 on bone growth and differentiation during embryogenesis and in adult tissues. They found that conditional elimination of Mdm2 in the osteogenic lineage resulted in multiple skeletal defects as a result of heightened p53 activity. Notably, in the absence of Mdm2-mediated regulation of p53, the induction of Runx2, the master regulator of osteoblast differentiation is greatly reduced in the osteoblast progenitors. As a result, there is excessive apoptosis early on in the caudal somites followed by reduced proliferation and maturation of osteoblasts at a later stage.

Godley et al. (1996) reported that mice expressing a wild type p53 transgene succumbed to end-stage renal disease. These mice presented with smaller kidneys having half the number of nephrons and glomerulosclerosis by E18.5. Although, from the rescue experiment it is safe to conclude that the phenotype we see is the consequence of p53 overexpression, our observations differ from those of Godley et al. (1996) in a number of aspects. Firstly, the UB^Mdm2^−/− kidneys were phenotypically recognizable as early as E13.5 as opposed to E18.5 with p53-transgenic kidneys. Secondly, we observed severe impairment of UB branching morphogenesis in the UB^Mdm2^−/− kidneys while branching defects were not reported in p53-transgenic mice. Thirdly, p53-transgenic mice manifested patterning defects such as ectopic expression of cRet, and absence of DB lectin and AQP-2 in the collecting ducts at E18.5. Albeit fewer in number, we still observe DB lectin-positive collecting ducts that are more centrally located, having failed to extend to the periphery. Also, AQP-2 protein is present in the UBs of UB^Mdm2^−/− mice. Finally, the p53 transgenic kidneys did not show any alteration of WT1 or LTA staining. In contrast, we observed reduced WT1 staining in newborn UB^Mdm2^−/− with severe depletion of renal progenitors. The presence of functional Mdm2 in the kidneys expressing transgenic p53 could

**Fig. 8.** Reduced cell proliferation accompanies the poor growth of the mutant UB^Mdm2^−/− kidneys. Double immunofluorescence for cytokeratin (green) and p-H3 (red) in E14.5 kidney sections of wild type (Panels A–C) and mutant (D–F) kidneys. Nuclei are counterstained with DAPI (blue). (G) A bar graph depicting quantitatively the reduced levels of cell proliferation in the conditional null mutant kidneys compared to the wild type and conditional heterozygous kidneys.

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**Table 1**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>A. All genotypes expected at 25%</th>
<th>B. Percent of progeny with various genotypes</th>
</tr>
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<tr>
<td>(Hoxb7 Cre^WT^; Mdm2^WT^) × Mdm2^WT^</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult n=37</td>
<td>Mdm2^+/+</td>
<td>Mdm2^WT^/+</td>
</tr>
<tr>
<td></td>
<td>0 (1:32)</td>
<td>4 (1:16)</td>
</tr>
<tr>
<td>p53^WT^/-</td>
<td>2 (1:16)</td>
<td>4 (1:8)</td>
</tr>
<tr>
<td>p53^-/-</td>
<td>0 (1:32)</td>
<td>3 (1:16)</td>
</tr>
</tbody>
</table>

**Table 2**

Survival to adulthood following concomitant deletion of Mdm2 and p53.

<table>
<thead>
<tr>
<th>Age</th>
<th>Mdm2^WT^+/+</th>
<th>Mdm2^WT^-/-</th>
<th>Cre Mdm2^WT^/+</th>
<th>Cre Mdm2^WT^-/-</th>
</tr>
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<tr>
<td>P21</td>
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<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>P0–P3</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>E14.5–17.5</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>E11.5–13.5</td>
<td>20</td>
<td>21</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>

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**Table 3**

Survival to adulthood following concomitant deletion of Mdm2 and p53.

<table>
<thead>
<tr>
<th>Age</th>
<th>Mdm2^WT^+/+</th>
<th>Mdm2^WT^-/-</th>
<th>Cre Mdm2^WT^/+</th>
<th>Cre Mdm2^WT^-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>P21</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>P0–P3</td>
<td>4</td>
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<td>2</td>
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<tr>
<td>E14.5–17.5</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>E11.5–13.5</td>
<td>20</td>
<td>21</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>

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explain why Godley et al. (1996) observed a less pronounced, late onset phenotype. The findings in the current study serve to emphasize the strict requirement for Mdm2 in the regulation of p53 during metanephrogenesis. We found that Mdm2 loss in the UB compartment resulted in the downregulation of Wnt9b in the UB and Lhx1 and Pax2 in nascent nephrons. The Wnt–β-catenin pathway is required for UB branching morphogenesis (Bridgewater et al., 2008; Marose et al., 2008) as well as for mesenchyme-to-epithelium conversion (Carroll et al., 2005). In a sequential step, Lhx1 mediates further differentiation of nephron progenitors to epithelial nephrons (Kobayashi et al., 2005). The transcriptional regulator, Pax2, is required for multiple steps of kidney development, including UB morphogenesis and growth and survival and differentiation of the renal progenitors (Brophy et al., 2001; Rothenpieler and Dressler, 1993; Torres et al., 1995). Chromatin immunoprecipitation studies coupled with next generation DNA sequencing (ChIP-Seq) using chromatin isolated from E15.5 mouse kidneys identified p53 binding peaks in the 5′-regulatory regions Pax2, Lhx1, and Wnt9b genes (Saifudeen Z., and El-Dahr SS, unpublished observations). These findings suggest that p53 target genes include important developmental renal regulators and point to a potential mechanism whereby dysregulation of p53 activity contributes to the observed phenotype.

The endowment of nephrons is determined primarily by the extent of UB branching during metanephrogenesis (al-Awqati and Goldberg, 1998; Basson et al., 2006; Nigam and Shah, 2009; Reidy and Rosenblum, 2009). Suboptimal nephron numbers could increase the risk of hypertension and predispose the affected individuals to renal insults later in life (Brenner et al., 1988; Keller et al., 2003; Poladia et al., 2006; Woods, 1999). Although mutations in the MDMP2 gene or
other components of the p53–Mdm2 pathway have not been reported in human renal dysgenesis, polymorphisms in the MDM2 gene promoter, recently reported to modulate cancer susceptibility in humans (Bond et al., 2004), cannot be ruled out. Future studies should focus on elucidating the role of Mdm2 in other compartments of the developing kidney (e.g., the metanephric mesenchyme and stroma) and on exploring the signaling pathways that are disrupted by loss of Mdm2-mediated inhibition of p53 during renal organogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.03.017.

Acknowledgments

This work was supported by NIH grants RO1-DK62250 and RO1-DK56264. S.H. was supported by a postdoctoral fellowship grant from the National Kidney Foundation (547240G1). We acknowledge the support of the Tulane Renal and Hypertension Center of Excellence and the Center for Gene Therapy. We thank Drs. Zubaida Saifudeen and Oliver Wessely for their inputs and insightful discussions about the project. We thank Drs. Cathy Mendelsohn, Greg Dressler, Thomas Carroll and Zubaida Saifudeen for the probes used for in situ hybridization.

References

