p53 Regulates Metanephric Development

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ABSTRACT

p53 is best known as a tumor suppressor that regulates cell-cycle, differentiation, and apoptosis pathways, but its potential role in embryonic development and organogenesis remains controversial. Here, p53−/− embryos bred on C57Bl6 background exhibited a spectrum of congenital abnormalities of the kidney and urinary tract, including ureteric bud (UB) ectopia, double ureters/collecting systems, delayed primary branching of the UB, and hypoplastic metanephroi. We observed ectopic UB outgrowth from the Wolffian duct (WD) in one third of p53−/− embryos. The prevalence of duplex was higher in embryos than in neonates, and ex vivo organ culture suggested that ectopic ureters can regress over time, leaving behind a dysplastic pole (“segmental dysgenesis”). Transgenic expression of dominant negative p53 or conditional inactivation of p53 in the UB but not in the metanephric mesenchyme lineage recapitulated the duplex phenotype. Mechanistically, p53 inactivation in the WD associated with enhanced sensitivity to glial cell line–derived neurotrophic factor (GDNF)-induced ectopic budding and potentiated phosphatidylinositol-3 kinase activation by GDNF in UB cells. Unlike several other models of UB ectopia, hypersensitivity of p53−/− WD to GDNF is not accompanied by reduced Sprouty-1 or anterior expansion of the GDNF domain. In summary, our data lend support for a restrictive role for p53 activity in UB outgrowth from the WD.


Organogenesis is dependent on a multitude of growth factors, signaling molecules, and transcription factors that temporally and spatially define a developmental program. Metanephric development is dependent on formation of the Wolffian duct (WD) and the adjacent metanephric mesenchyme (MM) from the intermediate mesoderm. In mice, the ureteric bud (UB) develops at embryonic day 10.5 (E10.5) from the caudal end of the WD. Inductive interactions between the UB and the MM ensure cell survival and subsequent onset of metanephrogenesis. Emergence of the UB from a specific site in the WD adjacent to the MM is controlled by the glial cell line–derived neurotrophic factor (GDNF)-cRet signaling pathway.1–6 GDNF is a growth factor that is secreted by the MM and binds to its receptor c-Ret and co-receptor GFRα1 that are expressed along the WD and UB tips. Stimulation of the GDNF-c-Ret pathway results in cell proliferation and chemotaxis.7,8 After the initial broad distribution of the GDNF field along the posterior half of the WD, the GDNF expression domain is progressively restricted to the caudal end of the WD in the immediate vicinity of the site of UB outgrowth9–11; however, the potential for bud emergence remains along the length of the WD, which continues to express the c-Ret/GFRα1 receptor/co-receptor. Evidence from mutant mouse models and metanephric organ culture implicates impaired restriction of the GDNF field and the GDNF/c-Ret signaling pathway in ureter duplica-
tion. Whereas loss of any component of this pathway results in renal agenesis, ectopic GDNF expression induces appearance of supernumerary buds, resulting in multilobed kidneys sometimes with additional ureters; therefore, inhibitors of GDNF/c-Ret expression or activity are necessary to restrict UB emergence to a specific site on the WD. Available data support two mechanistic scenarios on how ectopic c-Ret/GFRα1 activation might occur: (1) Anterior expansion of the GDNF field (i.e., anterior to the specified metanephric anlagen) as a result of loss of a GDNF repressor, such as Foxc1 or Slit2/Robo1; (2) increased responsiveness of the duct to GDNF from loss of antagonistic signals, such as Sprouty1 in the WD, or around the duct in the periureteral mesenchyme, such as Bmp4 or Agtr2. Both models are accompanied by ectopic GDNF-c-Ret activity. Thus, there is clear evidence of control at multiple levels to ensure normal metanephric development.

The tumor suppressor functions of p53 have been well characterized. Under stress stimulation, p53 transcriptionally regulates genes that control cell-cycle arrest, apoptosis, and DNA repair. All of these processes ensure elimination of genetically damaged cells from being propagated. In addition, p53 plays a role in promoting cell differentiation. Less well studied are its activation and role in embryonic development and non-stressed cells. p53-null mice are viable and show developmental abnormalities with incomplete penetrance on certain genetic backgrounds. This is in contrast to embryonic lethality observed in p53-depleted Xenopus embryos. Compensation by p53 family members p63 and p73 that are present in early mouse embryo has been suggested as a possible reason for the nonlethal phenotype.

Previous work from our laboratory has described the role of p53 in regulation of renal function genes in terminally differentiating renal epithelia. In this study, we found that loss of p53 also affects early events of kidney development. Metanephroi from conventional p53−/− mice exhibit ectopic UBs and duplex kidneys. Conditional deletion of p53, designed to eliminate p53 from either the WD or MM lineage, revealed the duplex ureter/kidney is phenocopied when p53 is inactivated/absent in the WD.

RESULTS

Developmental Pattern of p53 Gene Expression during Kidney Organogenesis

We analyzed p53 mRNA expression during metanephric development by in situ hybridization. p53 expression is ubiquitous in E10.5 mouse embryo, including the WD and surrounding mesenchyme (data not shown). At E11.5, p53 is abundantly expressed in the mesonephric tubules (Figure 1A, A and B). p53 mRNA expression is higher in the main UB, at the first branch (T) stage, and subsequently in the trunks and tips of UB branches than the surrounding condensed MM and stroma (Figure 1, C through E). After E13.5, p53 was expressed in nephron progenitors (Figure 1, E and G). By middle to late nephric development, at E16.5, p53 is highly expressed in the differentiating collecting ducts and nephrons (Figure 1, G and H). At postnatal day 1 (PN1), renal vesicles and comma- and S-shaped structures also express p53 mRNA, albeit at lower levels than collecting ducts.

Quantitatively, p53 expression in the kidney is developmentally regulated through embryonic growth and declines significantly from PN20 into adulthood, after cessation of postnatal nephric development (Figure 1F). We previously showed that nuclear p53 protein levels are higher in newborn mouse and rat kidneys than in adult by Western blot analysis, electrophoretic mobility shift assay, and chromatin immunoprecipitation.

p53 Null Mice Exhibit a Spectrum of Congenital Abnormalities of the Kidney and Urinary Tract

Double Ureters/Collecting System (Duplex).

In a previous study, we reported that p53−/− pups have a high prevalence of renal abnormalities, including persistent and ec-
topic proliferation, tubular dysgenesis, mislocalization of renal function genes, dysmorphic papillae, and hydronephrosis. 30,32 Embryonic analysis of these mice revealed double ureters in one third (three of nine) of p53−/− embryos and in nearly 17% (eight of 45) of heterozygous embryos, as compared with 0% (zero of 21) in wild-type (WT; Table 1; Figure 2). Figure 2D illustrates an example of p53−/− metanephroi that exhibit an ectopic bud invading a bi-lobed MM. Invasion of the abnormal MM by the ectopic ureter occurred anterior relative to the primary UB. During the culture period of 88 h, the distinct point of emergence of each UB from the WD disappeared and resembled a ureter bifurcation. Interestingly, the duplex UB in Figure 2F seemed to have fused at the base of anomalies observed in p53-deficient mice are listed in Table 1.

### Table 1. Spectrum of anomalies in p53-deficient mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conventional</th>
<th>Hoxb7-Cre/p53loxP</th>
<th>Pax3-Cre/p53loxP</th>
<th>Hoxb7-p53DN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−/−</td>
<td>+/+</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Hypoplasia*</td>
<td>7/7 (100%)</td>
<td>8/11 (73%)</td>
<td>3/17 (18%)</td>
<td>0/24</td>
</tr>
<tr>
<td>Duplex</td>
<td>3/9 (33%)</td>
<td>4/8/6 (5%)</td>
<td>0/52</td>
<td>0/74</td>
</tr>
<tr>
<td>Dysmorphic papilla/medulla defectb</td>
<td>24/53 (45%)</td>
<td>0/8</td>
<td>ND</td>
<td>12/44 (27%)</td>
</tr>
</tbody>
</table>

**a**Based on UB counts of cytokeratin-stained whole mounts.

**b**Papilla/medulla defect in PN1 through 3 sections.

**c**Four litters, 51 to 87% of WT.

**d**Includes −/− and +/− embryos, because −/− alone have duplexes and/or hydronephrosis.

Conditional Inactivation of p53 from the Nephric Duct Lineage Induces Ectopic Bud Emergence

To distinguish whether the double collecting system is a result of loss of p53 from the WD or MM, we conditionally deleted p53 either from the WD/UB (UBap53) or the mesenchyme (MMap53) by Cre-mediated recombination. To generate UBap53, we crossed p53loxP/loxP mice with mice expressing Cre-recombinase under the control of a 1341-bp fragment of the Ksp-cadherin promoter or the Hoxb7 promoter. UBap53 mice phenocopied the duplex observed in p53−/− mice (Figure 5, A and B). Incidence of duplex ureters/kidneys was six of (5%) 116 and four (5%) of 86 for Ksp-Cre– and Hoxb7-Cre–mediated deletion, respectively. In contrast, conditional deletion of p53 from the MM using Pax3-Cre−;p53loxP mice (MMap53) did not result in duplex ureter formation (zero of 74), indicating that loss of p53 from the MM is not sufficient to produce the duplex renal phenotype. Pax3-driven Cre expression was localized to the nephrogenic cord (E9.5) and to the MM adjacent to the nephric duct and UB at E10.5, but no expression was detected in the nephric duct. 33

Because Cre-mediated recombination is reportedly chimeric, we used Hoxb7 promoter–driven expression of dominant negative p53 (p53DN) as an additional method of inhibiting p53 activity in the WD/UB. p53DN contains the oligomerization domain and lacks the DNA-binding domain. 34 This miniprotein forms stable mixed multimers with endogenous WT p53, thereby inhibiting its functional interaction with DNA. Kidneys from newborn pups (PN1 through 3) from 10 founder lines were initially examined. Subsequent analyses were performed on lines from two founders that ex-
pressed the transgene in the collecting ducts (Figure 5). Kidneys from transgenic mice exhibit increased distribution of p53 staining in UBs and collecting duct epithelia (Figure 5, D and D inset), in contrast to basal levels exhibited in the non-transgenic kidney. Because the transgene construct also expresses green fluorescence protein (GFP) driven by the Hoxb7 promoter, serial tissue sections were stained for GFP, p53, and lectin Dolichos biflorus demonstrating coexpression in collecting ducts (Supplemental Figure 1). Transgene expression is maintained in differentiating collecting ducts, which stain positively for aquaporin 2 (AQP2; data not shown). As expected, tissue sections from nontransgenic mice were negative for GFP (Supplemental Figure 1).

Duplex ureters and multilobed kidneys occurred at a frequency of approximately 35% in Tg\(^{-}\) Hoxb7-p53DN mice (Figure 5E; Table 1). In addition, kidneys from these animals showed enlarged or duplex papillae (Figure 5H), ectopic extension of the nephrogenic zone, renal nodules, and pitting of the surface as a result of patterning defects (Figure 5, E’, F, and G). The double papilla stains positively for AQP2 (Figure 5H’). Collectively, our data demonstrate that the loss of p53 function in the WD/UB causes ureter duplication.

**Hypersensitivity of p53-Deficient WD to GDNF**

Occurrence of duplex collecting system in UB\(^{-}\)p53 but not in MM\(^{-}\)p53 suggested a WD/UB autonomous defect. We therefore hypothesized that varying p53 activity in the WD alters the sensitivity of WD to exogenous GDNF-induced budding *in vitro*. E11.5 WD respond to high-dosage GDNF (50 to 200 p53 and Kidney Development.

![Figure 2](image_url)  
**Figure 2.** Duplex kidney phenotype in conventional p53-null mice and delayed primary branch formation in p53\(^{-}\) metanephroi are shown. (A through F’) Growth and branching patterns of p53-null metanephroi ex vivo are examined. Metanephroi from p53\(^{+/+}\) and p53\(^{-/-}\) littersmates were cultured on transwell filters for 88 h, then stained with anti-cytokeratin antibody. At 0 h, the p53\(^{+/+}\) metanephroi (A and A’) show the T-shaped UB characteristic at E11.5. The UB in p53\(^{-/-}\) metanephroi has not yet branched (D and D’) at E11.5, resulting in fewer UB tips at 88h (C and C’ versus F and F’). (D) White arrows point to two UBs emerging at distinct points from the WD and invading the MM (black arrow). At 40 h (E), the two points of UB emergence seem to have merged into one, thus giving the appearance of a bifurcated ureter (E and F). Larger condensates in p53\(^{+/+}\) kidneys (black arrowheads, E and E’) than in p53\(^{+/+}\) littersmates (B and B’). Blind ectopic UBs (E and F, yellow arrowheads) are clearly visible at 40 and 88 h. (G through G’) Regression of an ectopic ureter over time (0 versus 88 h, G and G’) results in a single ureter with an abnormal lobulated kidney. (H) Periodic acid-Schiff–stained p53\(^{-/-}\) PN1 kidney section is shown. Red arrows point to the duplex lobes that are separated by a deep nephrogenic zone (boxed [H]; high magnification [H’]). (I) Section from a heterozygous kidney shows WT morphology.

![Figure 3](image_url)  
**Figure 3.** GDNF, c-Ret, and Spry1 mRNA levels and domains are unchanged in p53\(^{-/-}\) embryos. E10.5 litters from p53\(^{+/+}\) pairings were dissected and used for *in situ* hybridization. AP, anterior-posterior; h, hind-limb bud.
ng/ml) by growing multiple c-Ret+ buds along the duct, as demonstrated in Figure 6, B and C.12 In comparison, a low dosage of GDNF (10 ng/ml) does not elicit bud formation in WT WD (Figure 6A). p53+/− and Hoxb7–p53DN−/− WD, however, grow supernumerary buds in response to low-dosage GDNF treatment (Figure 6, D and E). The number of tips was consistently lower in p53+/− (yellow bar) versus p53+/+ metanephric (purple bar), with the null metanephroi showing 30% fewer tips than the WT.

Because bud emergence is reportedly preceded by a spurt of cell proliferation13 and because p53 regulates cell proliferation, we examined proliferation in p53-null ducts. Because bud formation is visible by 48 h of GDNF treatment, we assayed for proliferation 24 h after treatment, before when buds are usually visible. Phospho-histone 3 (P-H3-S10) staining did not show increased proliferation in p53-null nephric ducts on

Figure 4. Hypoplasia in p53−/− kidneys is shown. (A) In situ hybridization for Spry1 was done on E12.5 metanephroi. Branch and tip formation are lagging in the p53−/− metanephros in comparison with the age-appropriate branching observed in a p53+/+ littermate. There was no change in Spry1 mRNA levels. (B) Decreased proliferation in p53-null kidneys was visualized by phospho-H3 antibody staining compared with kidney section from WT littermate. (C) UB tip numbers were counted in four litters from p53−/− pairings. (D) The number of tips was consistently lower in p53−/− (yellow bar) versus p53+/+ metanephric (purple bar), with the null metanephroi showing 30% fewer tips than the WT.

Figure 5. WD/UBp53−/− mice have kidneys with double papillae. (A, B, and D through H) p53 was conditionally deleted in the WD/UB by crossing Ksp-cadherin–driven (A) or Hoxb7-driven (B) Cre transgenic mice to p53floxed mice or by expressing p53DN driven by Hoxb7 promoter (D through H). (C) Hoxb7-p53DN construct. p53 cDNA encoding a miniprotein (amino acids 1 through 14 and 302 through 390) was inserted into a Hoxb7-IRES-EGFP construct to drive expression of the mutant protein (p53DN) in WD lineage. (D and D’) Immunofluorescence staining with anti–p53-P-ser392 is shown. Section from a transgenic mouse kidney expressing p53DN shows intense staining in UB trunks and branches (D periphery, and inset) and deeper collecting ducts. Section from a nontransgenic mouse kidney shows much lower intensity of staining, representing detection of endogenous levels of p53-P-ser392 (D’, magnified inset). (E) Transgenic mice expressing this miniprotein exhibit duplex ureters. (E’) Periodic acid-Schiff staining of a section reveals an internalized nephrogenic zone (arrow). (H and H’) The double papilla (H, black arrows) stains positively for AQP2 (H’, magnified). (F and G) Hoxb7-p53DN gain-of-function phenotype: Extrarenal nodules (F) and pitted surface (G).
comparison to WT ducts (Supplemental Figure 2), suggesting that ectopic bud formation in p53-null ducts is not a proliferation defect. We may have missed differences in proliferation as a result of the time point that was assayed, or these differences may be subtle. Nevertheless, our data demonstrate that p53 activity in the WD is required and tightly regulated to allow normal UB outgrowth.

**p53 Loss Potentiates Phosphatidylinositol-3 Kinase Activation by GDNF in UB Cells**

To explore the mechanism for increased sensitivity of p53-null ducts to GDNF, we used a cell-culture system. UB cells were transfected with p53 small hairpin RNA (shRNA) plasmid or small interfering RNA (siRNA) and treated with GDNF/ GFRα1 before harvest. Loss of p53 resulted in dramatic up-regulation of phospho-Akt, measured as readout of phosphatidylinositol-3 kinase (PI3K) activity (Figure 7A), without any change in total Akt levels. Upregulation in p-Akt levels was dependent on the extent of p53 knockdown. We observed a variable two- to 10-fold decrease in p53 protein levels and corresponding two- to six-fold increase in p-Akt in comparison with negative control samples that were transfected with a scrambled siRNA. Next we measured phosphatase and tensin homolog (PTEN) levels, because PTEN is positively regulated by p53 and inhibits P-Akt production via phosphatidylinositol 3, 4, 5-triphosphate. Surprisingly, PTEN levels did not decrease with p53 knockdown. Phospho-extracellular signal-regulated kinase 1/2 levels were not measured in this study. Protein levels were normalized to β-actin or total Akt. These data provide biochemical evidence that p53 antagonizes the GDNF→c-Ret→PI3K pathway.

**Figure 6.** p53-deficient nephric ducts exhibit increased sensitivity to GDNF. (A through C) WT nephric ducts grow ectopic c-Ret UBs in response to exposure to high dosage (>50 ng/ml) GDNF (B and C). Exposure to subthreshold dosage (10 ng/ml) does not elicit bud growth (A). (D through E’) Subthreshold dosage exposure with GDNF of p53-deficient ducts induces multiple ectopic bud formation in mutant (D’ and E’) ducts but not in ducts from WT littermates (D and E, respectively). (F and F’). WT ducts treated with p53-inhibitor Pifithrin also respond to 10-ng/ml dose of GDNF with ectopic buds (F’); the contralateral DMSO-treated duct does not (F). (G through I) p53 stabilization by treatment with 5 μM (H) or 10 μM (I) Nutlin3a renders the duct refractory to 50 ng/ml GDNF, whereas DMSO-treated duct responds appropriately (G).

**Figure 7.** (A) p53 knockdown by shRNA potentiates PI3K activation by GDNF in UB cells. UB cells were transfected with GFP, scrambled, or p53 shRNA plasmids for 72 h. Transfected cells were treated with GDNF+GFRα1 20 h before harvesting for Western blot analyses. Decrease in p53 levels is accompanied by increase in P-Akt, without an increase in total Akt. PTEN levels did not decrease with p53 knockdown. Fold differences in levels are normalized for total Akt. (B) Model of p53 function in WD is shown. p53 opposes pathways activated by growth stimuli, such as GDNF→c-Ret→PI3K. Antagonism may occur at more than one site along the pathway; p53 regulates expression of genes such as PTEN that antagonize PI3K function, or p21/Cip1 and FAK, inhibitors of proliferation and cell migration, respectively. p53 negatively regulates expression of PI3KCA, the catalytic subunit of PI3K.
DISCUSSION

In this study, we provide evidence that p53 function is required for early events of kidney development. p53 activation in response to genotoxic stressors is well documented. Initial reports on p53-null mice did not credit p53 with a developmental role; however, subsequent analyses of the mouse models showed genetic background–dependent anomalies, including female exencephaly, craniofacial and ocular abnormalities, embryonic lethality, and defects in spermatogenesis. We found that loss of embryonic p53 caused maldevelopment of the kidney and urinary tract in mice on a C57BL6 background.

We previously described a role for p53 in regulating expression of renal function genes in terminally differentiating renal epithelium. Observation of duplex renal papillae in PNI kidneys from p53-null neonates prompted us to analyze the effects of p53 deficiency on early kidney development. Up to one third of p53−/− embryos on C57BL6 background exhibit a unilateral duplex phenotype. Whereas conditional UBp53DN mice exhibit the duplex ureter/kidney phenotype, the incidence of duplex ureters is four- to six-fold lower than that observed in conventional p53−/− mice. Lower penetrance of the duplex in conditional versus conventional p53-null mice may be an effect of different genetic backgrounds. Alternatively, the level of p53 knockdown achieved with Cre-mediated recombination may not be robust or is compensated by family members p73/p63. To address this possibility, we overexpressed a p53DN miniprotein in the WD under the control of the Hoxb7 promoter (UBp53DN). One third of UBp53DN mice exhibit duplex ureters/kidneys, as observed in conventional p53-null embryos. p53DN forms stable mixed multimers with endogenous WT p53, thereby inhibiting its functional interaction with DNA and effectively blocking WTp53 function to levels that may not have been achieved in the Cre-recombination models. Alternatively, p53DN may have a gain-of-function effect that allows duplex formation by a p53-independent mechanism (e.g., by antagonizing p73/p63 activity). The latter effect may explain the extrarenal nodules and pitting of the kidney surface as a result of patterning defects observed in UBp53DN mice.

Several current models of supernumerary budding cite aberrant expression or signaling along the GDNF-c-Ret-Spry1 pathway as the primary defect leading to ectopic budding; however, there are other genetic models, such as conditional β-catenin−/WD and Lim1 mice, that do not. In our model of ectopic budding, we did not observe an expanded GDNF domain or changes in c-Ret or Sprouty1 expression. Unlike the β-catenin and lim-1 models, we found p53 deficiency in the WD/UB lineage does confer enhanced sensitivity to GDNF-induced budding but not via increased proliferation. Differential cell adhesion has been postulated as a cause of ectopic bud formation. p53 is a known regulator of adhesion molecule expression such as E-cadherin, as well as of adhesion-associated molecules such as Perp. Comparing adhesion and migration differences in cells from p53-mutant ducts, although challenging, will offer much insight into mechanisms of UB outgrowth.

How might increased sensitivity of the WD to low-dosage GDNF occur in the absence of functional p53? Our data are consistent with a model in which p53 deficiency or inactivation releases inhibitory input on cell adherence/migratory/proliferative signaling mediated by the Ret tyrosine kinase receptor in the WD (Figure 7); this in turn results in emergence of accessory UBs and abnormal branching patterns. Even though we did not find decreased PTEN levels with p53 knockdown that could explain increased P-Akt levels, it should be noted that PTEN activity is regulated by posttranslational modifications and may be the case in this system. Another possible mechanism involves variability in heparin sulfate proteoglycans levels that would alter activity of signaling proteins such as GDNF, fibroblast growth factors, bone morphogenic proteins, and WNTs. p53 modulates expression of protein regulators of extracellular heparin sulfate, such as heparanase and extracellular heparin sulfate 6-O-endosulfatases.

Transgenic mice overexpressing WT p53 in the kidney exhibit differentiation defects in the UB. A consequence of abnormal signaling to the MM by the UB is increased mesenchymal apoptosis and decreased mesenchyme to epithelial transition, resulting in hypoplastic kidneys. This is probably the case in p53-null kidneys as well, which are also hypoplastic. This phenotype was surprising, considering the p53-null nephric duct has ectopic UB outgrowth; however, as stated already, ectopic UB formation is not a consequence of increased proliferation. p53-null metanephroi at E11.5 show delayed T formation that may contribute to a decreased number of UB tips. In addition, p53-null kidneys exhibit decreased proliferation that would also impair UB morphogenesis.

Future studies will define additional mechanisms of p53 function in metanephric development, which in its absence results in WD hypersensitivity to growth stimulation but growth inhibition in the mesenchyme.

CONCISE METHODS

Mice

Conventional p53−/− mice (trp53−/−) on C57Bl6 background were purchased from the Jackson Laboratory, and heterozygotes were used as breeder pairs. A neomycin cassette replaces exons 2 through 6 and eliminates p53 mRNA synthesis. Conditional lineage-specific p53 deletion was achieved by crossing p53loxP/loxP mice (gift from NCI/gene generated by Anton Berns), with nephric lineage-specific Cre expressers Ksp-cadherin-Cre (gift from Peter Igarashi), Hoxb7-Cre (gift from Carlton Bates), and Pax3-Cre (gift from Feng Chen and Jonathan Epstein). Transgenic Hoxb7-p53DN-IRES-GFP mice with targeted expression of p53DN in the WD/UB-derived structures (Hoxb7-p53DN) were generated at the Tulane Transgenic Mouse Facility. p53DN encodes a miniprotein containing amino acids 1 through 14 fused to the last 89 residues of murine WT p53. The p53 miniprotein cDNA construct was a gift from M. Oren.
cDNA was amplified with forward and reverse primers containing KpnI restriction sites at the 5’ overhang. The PCR product was digested with KpnI and subcloned into KpnI-digested Hoxb7-IRES-EGFP plasmid (gift from Carlton Bates). Analysis was done on progeny from 10 founders. All animal experiments were approved by the Tulane University Institutional Animal Care Committee.

**Organ Culture**

E11.5 WDs or E12.5 metanephroi were cultured on Transwell filters in DMEM/F12 medium with 10% FCS at 37°C/5% CO2. For branching morphogenesis studies, metanephroi were cultured for 48 to 72 h and stained, and tips/branch points were counted. For GDNF sensitivity studies, metanephroi were treated with low-dosage GDNF (10 ng/ml; R&D Systems) in the presence of p53 inhibitor PFTα (Sigma) or DMSO. To stabilize p53 levels, Nutlin-3, a small-molecule inhibitor of MDM2 (10 μM; Cayman Chemicals), was added to the medium with high-dosage GDNF (50 to 100 ng/ml). Media containing GDNF and PFTα/Nutlin-3/DMSO were replaced every 24 h.

**Immunofluorescence Staining**

**Whole Mount.** Metanephric explants were fixed in ice-cold methanol for 10 min, washed twice in PBS/0.1% Tween for 30 min each, and incubated with primary antibody overnight at 4°C. Monoclonal anti-pancytokeratin antibody (Sigma; 1:200), and Lotus Tetragonolobus agglutinin (Vector Laboratories; 1:100) were used to stain collecting ducts and proximal tubules, respectively. A polyclonal anti-Pax2 antibody (1:200; Tor Laboratories; 1:100) were used to stain collecting ducts and proximal tubules, respectively. A polyclonal anti-Pax2 antibody (1:200; Zymed) was used to stain the MM and UB in urogenital blocks. After multiple washes in PBS/Tween, explants were incubated with Alexa-fluor–tagged secondary antibody overnight at 4°C, washed, and mounted on a slide with fluorescence mounting medium (Dako). Images of stained tissue were captured using an Olympus BX51 fluorescence microscope.

**Sections.** Metanephric E11.5 embryos were fixed in 10% formalin and processed for paraffin embedding and sectioning. Immunostaining was done as described previously. Antibodies for the following proteins were used: p53-P-Ser392 (Cell Signaling; 1:50), GFP (Invitrogen), Dolichos biflorus agglutinin (Sigma; 1:40), and AQP2 (Santa Cruz Biotechnology; 1:400).

**In Situ Hybridization**

Section and whole-mount ISH were performed as described at http://www.hhmi.ucla.edu/derobertis. E10.5 to E11.5 mouse embryos or E12.5 kidneys were fixed overnight in 4% formaldehyde in PBS, then dehydrated in methanol and stored at −20°C until use. Digoxigenin-dUTP–labeled RNA probes were used at 0.5 μg/ml. Alkaline phosphatase–conjugated anti-digoxigenin Fab fragments were used at 1:5000. Color reactions were carried out from overnight (c-Ret, p53, and Spry1) to several days (GDNF). Embryos were photographed in glycerol. The following probes were used for whole-mount ISH: p53 (clone ID 1349046, antisenseT3/senseT7; Invitrogen Clone Collections), c-Ret,59 GDNF,60 and Sprouty1.15

**Reverse Transcriptase–PCR**

Quantitative reverse transcriptase–PCR was performed on RNA from embryonic kidneys. Real-time primers for p53 were ordered from Applied Biosystems (Taqman Gene Expression Assay, ID Mm01731287_m1). β-Actin primers and probe sequences are as follows: Forward 5’-ACGGCCAGGTCTACATTTG; reverse 5’-CAAGAAGGAAGCTGGAAGG; and probe [5HEX]-CAAC-GAGGGTTCCGATGCC.

**Western Blotting**

UB cells were transfected with p53-shRNA plasmid (SuperArray Biosciences) or siRNA (ID s75472 and s75474; Applied Biosystems) for 72 h. Cy3-labeled negative control siRNA (AM4621; Applied Biosystems) or scrambled negative control plasmid (SuperArray) was used as control. Twenty hours before harvesting proteins, cells were treated with recombinant GDNF 50 ng/ml and GFRα1 100 ng/ml (R&D Systems) as described previously.61 Proteins were harvested in RIPA buffer supplemented with protease and phosphates inhibitors, resolved on 4 to 20% SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting with the following antibodies: p53 (SC6243; Santa Cruz Biotechnology), P-473-Akt and Akt (Cell Signaling), and PTEN (Cell Signaling).

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**DISCLOSURES**

None.

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