A p53-Pax2 Pathway in Kidney Development: Implications for Nephrogenesis

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Abstract
Congenital reduction in nephron number (renal hypoplasia) is a predisposing factor for chronic kidney disease and hypertension. Despite identification of specific genes and pathways in nephrogenesis, determinants of final nephron endowment are poorly understood. Here, we report that mice with germ-line p53 deletion (p53−/−) manifest renal hypoplasia; the phenotype can be recapitulated by conditional deletion of p53 from renal progenitors in the cap mesenchyme (CMp53−/−). Mice or humans with germ-line heterozygous mutations in Pax2 exhibit renal hypoplasia. Since both transcription factors are developmentally expressed in the metanephros, we tested the hypothesis that p53 and Pax2 cooperate in nephrogenesis. In this study, we provide evidence for the presence of genetic epistasis between p53 and Pax2: a) p53−/− and CMp53−/− embryos express lower Pax2 mRNA and protein in nephron progenitors than their wild-type littermates; b) ChIP-Seq identified peaks of p53 occupancy in chromatin regions of the Pax2 promoter and gene in embryonic kidneys; c) p53 binding to Pax2 gene is significantly more enriched in Pax2−/-expressing than non-expressing metanephric mesenchyme cells; d) in transient transfection assays, Pax2 promoter activity is stimulated by wild-type p53 and inhibited by a dominant negative mutant p53; e) p53 knockdown in cultured metanephric mesenchyme cells down-regulates endogenous Pax2 expression; f) reduction of p53 gene dosage worsens the renal hypoplasia in Pax2−/- mice. Bioinformatics identified a set of developmental renal genes likely to be co-regulated by p53 and Pax2. We propose that the cross-talk between p53 and Pax2 provides a transcriptional platform that promotes nephrogenesis, thus contributing to nephron endowment.

Introduction
Mammalian kidney development is dependent on reciprocal inductive interactions between the metanephric mesenchyme and the ureteric bud (reviewed in [1]). Nephrons arise exclusively from the nephron progenitor cell population that is adjacent to the ureteric bud, and is referred to as the cap mesenchyme (CM) [2,3]. Conversion of the progenitor CM cells to nephron epithelia requires co-ordinated expression of key transcriptional regulators that drive expression of distinct gene targets [4–6]. The CM expresses key transcription regulators that include Six2, Pax2, Eya1, Sall1, Wt1, Meox, and Cited1 [1,7], reviewed in [8]). These genes, either individually or combinatorially, drive cell-survival, self-renewal, differentiation and nephrogenesis.

The paired box (Pax) gene family of transcription factors consists of nine members that are grouped into four classes based on their DNA binding specificities and developmental expression [9,10]. Of the Pax2/5/8 sub-group, expression of Pax2/8 is essential and sufficient to induce nephric lineage and nephric duct morphogenesis, and both genes are required for nephron differentiation [11,12]. Pax2−/- mice exhibit anomalous development of the midbrain, the cerebellum [13] and the optic nerve [14] and renal agenesis [15]. Pax2 is a known determinant of ureteric bud (UB) branching and nephron number and Pax2 haplo-insufficiency in mice or humans is associated with renal hypoplasia [16,17]. Pax2, in concert with Hox11 and Eya1, is required for expression of Six2 and GDNF [18,19], essential regulators of nephron progenitor cell population and branching morphogenesis, respectively. Pax2-null mice lack GDNF expression and have decreased Six2 expression [15,20]. Wt1 is another important Pax2 target in the developing nephron. Decreased Wt1 expression was demonstrated in vivo in hypoplastic kidneys of heterozygous Pax2 mutant mice [21].

The role of p53 in maintaining genomic stability is well-documented. In the absence of stress, p53 levels in adult cells are low. Stabilization and activation occur upon genotoxic or oncogenic stress, resulting in a transcriptional program that activates cell cycle arrest, apoptosis or senescence [22–24]. Induction of differentiation programs is also viewed as a tumor suppressor function of p53 since this results in removal of the cell from the proliferative pool [22]. p53 represses expression of...
defects in spermatogenesis, severe ocular abnormalities, and background dependent developmental abnormalities, such as tumors early (renal hypoplasia in phenotype [40]. We have described duplex ureter formation and embryos upon p53 depletion [38,39]. Compensation by p53 family Developmental abnormalities with incomplete penetrance in mice higher in differentiating tissues. Although autonomous role for p53 in the CM in nephron formation. At P0, nephrons in sections showed fewer immature nephrons in the nephrogenic Cap Mesenchyme proximal tubules in E11.5 kidneys cultured for 72 h (Fig. 1A, B). kidneys revealed 50% fewer and less complex LTA-positive structures than wild-type kidneys (p<0.005). WT1 staining of E17.5 kidney targets of p53 transcriptional function in the developing kidney, we 

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p53 \text{ expression is ubiquitously high during early embryonic life}\ [31]. \text{ After mid-gestation, } p53 \text{ expression is tissue-specific and higher in differentiating tissues. Although } p53\text{-null mice develop tumors early (} \sim 4-6 \text{ mo), they are viable and show genetic background dependent developmental abnormalities, such as defects in spermatogenesis, severe ocular abnormalities, and } \sim 20\% \text{ female embryonic lethality due to exencephaly [32-37]. Developmental abnormalities with incomplete penetrance in mice are in contrast to embryonic lethality observed in Xenopus embryos upon } p53 \text{ deletion [38,39]. Compensation by } p53 \text{ family members, } p63 \text{ and } p73, \text{ that are present in early mouse embryo has been suggested as a possible reason for the non-lethal phenotype [40]. We have described duplex ureter formation and renal hypoplasia in } p53^{-/-} \text{ mice [41], as well as impaired terminal differentiation of renal epithelia [42]. } p53 \text{ deletion also impairs differentiation of skeletal muscle cells, various hematopoietic cell-lines, thyroid cells, keratinocytes, oligodendrocytes [22,43,44], neuronal maturation, axon outgrowth and regeneration [45]. In contrast, excess } p53 \text{ activity, achieved by deletion of its negative regulator MDM2, results in embryonic death at peri-implantation stage that can be rescued by } p53 \text{ deletion [46]. We recently showed that conditional } Mdm2 \text{ deletion in the nephric duct lineage derived ureteric epithelium results in severe hypoplastic kidneys as a result of excessive apoptosis, decreased proliferation of the ureteric epithelium, and repression of the nephron inducer Wnt9b [47]. Inhibition of UB branching and concomitant nephrogenesis defects were rescued by genetic deletion of } p53. \text{ Like } Pax2, p53 \text{ is expressed in the intermediate mesoderm and urogenital ridge and later in development in the mesonephros and metanephros [11,41]. In this study, we demonstrate that } p53 \text{ and } Pax2 \text{ are epistatic and that } p53 \text{ is an endogenous activator of } Pax2 \text{ gene expression during kidney development. These findings led us to propose that down-regulation of } Pax2 \text{ contributes to renal hypoplasia in } p53\text{-null embryos.} \n
\section*{Results}

\subsection*{Nephron Deficit in Germ-line \( p53^{-/-} \) Mouse Kidneys is Recapitulated by Conditional \( p53 \) Deletion from \( Six2+\) Cap Mesenchyme}

We previously described a defect in UB branching morphogenesis in \( p53^{-/-} \) mice [41]. Additional characterization of \( p53^{-/-} \) kidneys revealed 50% fewer and less complex LTA-positive proximal tubules in E11.5 kidneys cultured for 72 h (Fig. 1A, B). Mutant kidneys exhibit significantly fewer LTA+ structures than wild-type kidneys (p<0.005). WT1 staining of E17.5 kidney sections showed fewer immature nephrons in the nephrogenic zone as well as a reduction of mature glomeruli in the inner cortex (Fig. 1C). Notably, the presence of renal hypoplasia and fewer nephrons in \( p53^{-/-} \) metanephros is recapitulated in mice with conditional \( p53 \) deletion from \( Six2+\) Cap mesenchyme (CM\(^{p53^{-/-}}\)) (Fig. 1D), which are the nephron progenitor cells, suggesting an autonomous role for \( p53 \) in the CM in nephron formation. At P0, 6/8 (75%) examined CM\(^{p53^{-/-}}\) kidneys were hypoplastic compared to wild-type littermate kidneys (n = 8) (Fig. 1D). Persistence of hypoplasia post-natally suggests that the phenotype cannot be attributed simply to developmental delay. Lhx1 staining marks the differentiating nascent nephron population. Note fewer generations of Lhx1+ nascent nephrons in CM\(^{p53^{-/-}}\) kidneys (Fig. 1D, x10 images). Results of microarray analysis revealed that \( Pax2 \) expression is down-regulated in \( p53^{-/-} \) kidneys versus \( p53^{+/+} \) (p = 0.037; n = 5 animals/group). This finding prompted us to test the hypothesis that \( Pax2 \) is a \( p53 \) target gene. The details of the microarray will be published elsewhere (manuscript in preparation).

\subsection*{\( P53 \) and \( Pax2 \) have overlapping expression in the developing kidney}

\( p53 \) expression is developmentally regulated in the kidney [41]. At early stages (E11.5–12.5) \( p53 \) expression is ubiquitous; however, it is progressively restricted to differentiating renal epithelia as postnatal development proceeds and declines to non-detectable levels in adult kidneys. \( Pax2 \) expression is also developmentally regulated [11]. \( Pax2 \) is expressed throughout nephrogenesis in the \( Six2+\) CM, in pretubular aggregates (PTA) and renal vesicles (RV) which are the differentiating nascent nephrons, in distal portions of the S-shaped body of the maturing nephron and in the ureteric tips/collection ducts [12]. \( p53 \) expression is more widespread in the mesenchyme than \( Pax2 \), showing expression in metanephric and stromal mesenchyme. Immunofluorescence staining demonstrated that \( p53 \) is expressed in the \( Pax2+\) CM as well as in the UB (Fig. 2A).

\subsection*{Decreased \( Pax2 \) Expression in \( p53^{-/-} \) Metanephiroi}

To validate our microarray findings, we examined \( Pax2 \) gene expression in kidneys from \( p53^{-/-} \) and \( p53^{+/+} \) littermates. In situ hybridization revealed lower \( Pax2 \) mRNA levels (Fig. 2B) in 4/11 (36%) null embryos, in line with the variable severity of renal hypoplasia in \( p53^{-/-} \) mice [41]. To confirm the \( Pax2 \) ISH data, QPCR was done on RNA from individual E15.5 kidney pairs from wild-type (n = 4 pairs) and germ-line \( p53^{-/-} \) embryos (n = 4 pairs). The scatter-plot in Fig. 2C shows a significant decrease in \( Pax2 \) gene expression in individual mutant kidney pairs. In addition to decrease in \( Pax2 \) transcript, immunofluorescence staining with \( Pax2 \) antibody of wild-type and mutant E15.5 kidney sections showed significantly decreased expression of \( Pax2 \) protein in both ureteric and mesenchyme lineages in \( p53^{-/-} \) kidneys (Fig. 2D, E). Quantitation of staining intensity was done using Slidebook software and is shown graphically (Fig. 2E). Since kidneys with conditional \( p53 \) deletion from CM cells recapitulate the nephron deficit phenotype of global \( p53 \)-null kidneys, we examined \( Pax2 \) expression in CM\(^{p53^{-/-}}\) kidneys. Intensity of \( Pax2 \) staining was markedly reduced in the cap mesenchyme of E12.5 CM\(^{p53^{-/-}}\) kidneys in comparison to wild-type CM (Fig. 3A) indicative of lower \( Pax2 \) protein levels. Moreover, \( Pax2 \) mRNA is \sim 40% lower in E15.5 CM\(^{p53^{-/-}}\) kidneys (Fig. 3B).

\section*{ChIP-Seq Identification of \( p53 \) Bound to Multiple Regions in the \( Pax2 \) Promoter in Developing Kidneys}

\textit{In silico} analysis of the \( Pax2 \) promoter revealed several putative consensus \( p53 \) binding sites, suggesting \( p53 \) may directly regulate \( Pax2 \) expression during kidney development. To identify putative targets of \( p53 \) transcriptional function in the developing kidney, we performed ChIP-Seq using the Genome Analyzer (Illumina) on chromatin from E15.5 kidneys. The complete annotated data set of genes associated with \( p53 \)-bound regions will be reported.
Figure 1. Nephron deficit in germ-line $p53^{-/-}$ and $CM^{p53^{-/-}}$ kidneys. A, B) $p53^{-/-}$ kidneys exhibit 50% fewer and less complex LTA-positive proximal tubules. Metanephroi were harvested at E11.5, cultured on trans-well filters for 72 h and stained for cytokeratin and LTA. LTA counts were averaged from kidneys collected from at least 4 embryos. Mutant kidneys exhibit significantly fewer LTA+ structures than wild-type kidneys ($p<0.005$). C) E17.5 kidneys were harvested from embryos from $p53^{+/+}$ crosses, formalin-fixed and sectioned for immunostaining (Methods). Sections were stained with WT1 and cytokeratin antibodies. $p53^{-/-}$ kidneys show paucity of WT1 stained nephrons. D) Kidneys from mice with conditional $p53$ deletion from Six2+ cap mesenchyme ($CM^{p53^{-/-}}$). Hypoplasia persists post-natally, shown in P0 kidneys, top panels at 4x. Bottom panels (x10) show fewer Lhx1-positive nascent nephrons (red). At P0, 6/8 (75%) examined $CM^{p53^{-/-}}$ kidneys were hypoplastic compared to wild-type littermate kidneys ($n=8$).

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ChIP-PCR validation of several genes identified in the ChIP-Seq was recently published [48]. Figure 4 shows the ChIP tracks of p53 enrichment at the Pax2 gene. Tracks for the Input (pre-ChIP) sample show non-specific background that was subtracted from p53-IP sample prior to peak-calling. p53 is enriched at the Pax2 proximal promoter (Region 1) and at an intronic region (Region 2) (Fig. 4A). Region 1 extends from −1200 bp to −60 bp relative to the RefSeq annotated transcription start site (TSS) (http://www.ncbi.nlm.nih.gov/nuccore/NM_011037), with peak sequenced fragment density at −844 bp (Fig. 4A, green bars). p53 binding within the gene body encompasses a −400 bp region and overlaps intron2 and exon3, with peak fragment density in intron2. Two additional regions of

![Figure 2. Decreased Pax2 expression in p53−/− embryonic kidneys. A) p53 expression overlaps that of Pax2 in the developing kidney. Immunofluorescence staining was done on E15.5 kidney sections to visualize Pax2 and p53 protein expression. p53 co-localizes with Pax2 in the cap mesenchyme (CM) and in the ureteric tip (UB). B) In situ hybridization. Decreased expression of Pax2 mRNA at E12.5. C) QPCR. Pax2 mRNA is significantly lower in germ-line p53−/− kidneys. QPCR was done on RNA from individual E15.5 kidney pairs from wild-type and p53−/− embryos (n = 4). D) Pax2 protein in E15.5 kidneys, detected by IF staining. Both wild-type and mutant kidney sections were processed for IF staining simultaneously and images captured at identical exposure setting. After normalizing intensity to that in wild-type, images were converted to a heat-map to demonstrate differential Pax2 staining between wild-type and mutant kidneys. High intensity staining corresponds to red/yellow areas and lower intensity corresponds to green/blue. Top panels at x4 and bottom panels at x20. E) Quantitation of staining intensity was done using Slidebook software and is shown graphically. UB, ureteric bud/tips; CM, cap mesenchyme; Nn, nascent nephrons including pretubular aggregates and renal vesicles. doi:10.1371/journal.pone.0044869.g002](http://www.plosone.org/doi/10.1371/journal.pone.0044869.g002)
High p53-occupancy were found at Regions 3 and 4 at \(2.15\) kb and \(2.87\) kb, respectively, from the \(Pax2\) TSS (Fig. 4A).

Multiple p53 binding motifs were identified in and around p53-occupied regions, thus validating specificity of the ChIP (Fig. 4C). Interestingly, while sequence conservation in mammals is high as expected for proximal promoter and gene sequences at Regions 1 and 2, respectively, non-genic distal p53-enriched regions 3 and 4 also show high sequence conservation (Fig. 4B). Non-genic conserved regions are postulated to function as transcriptional regulators [49]. Indeed, transcription factor binding site search (Genomatix) revealed p300 and CTCF binding sites, which are suggestive of enhancer function, flanking p53 binding sites in the \(2.83\) kb p53-enriched region (region 4). Therefore, we sub-cloned this \(2\) kb region upstream to SV40 minimal promoter in pGL3-promoter-luciferase reporter plasmid. However, reporter activity was not enhanced either at baseline or in response to p53 in metanephric mesenchyme mK4 cells (data not shown). Enhanced \(Pax2\) expression in response to p53 via this putative enhancer-fragment in other cell types (e.g., neural or intermediate mesoderm) cannot be ruled out. Collectively, these data strongly suggest that \(Pax2\) is a physiological target of p53-mediated transcriptional regulation.

**Differential p53 Binding at \(-844\) in \(Pax2\) Promoter in mK4 (Pax2+) versus mK3 (Pax2-) Cells**

\(Pax2\) is expressed in induced metanephric mesenchyme derived mK4 cells, but not in mK3 cell line that represent early uninduced mesenchyme cells [50]. Both cell lines express p53 (Fig. S1). To
Figure 4. p53 occupancy at the *Pax2* gene locus in E15.5 kidneys identified by ChIP-Seq. A) Genomic view of p53 occupancy at the *Pax2* gene locus identified by ChIP-Seq and visualized using the Integrated Genome Browser (IGB). Orange bars denote p53 enriched regions after background (Input) subtraction. Green bars show MACS peaks which represent points of highest density of sequenced fragments within the orange interval. Peak 1 and 2 are in the proximal promoter and in intron2-exon3, respectively. Two additional peaks were identified far distal to the *Pax2* gene at ~14 kb (peak 3) and ~83 kb (peak 4) from the transcription start site. Yellow box shows region validated for p53-enrichment by ChIP-PCR in Fig. 5. B) High sequence conservation in mammals of regions encompassing p53-enriched areas, including the non-genic distal regions 3 and 4, visualized in the UCSC genome browser (http://genome.ucsc.edu). C) Red bars show location of p53 binding motifs in and around p53-occupied regions 1 and 2 in the *Pax2* promoter/gene. Although p53 binding sites are broadly scattered across the entire region including the intervening region between regions 1 and 2 (see Fig. S3), p53 occupancy occurs at specific regions.

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determine whether p53 is differentially bound at the Pax2 proximal promoter in Pax2-expressing versus non-expressing cells, chromatin was immunoprecipitated from both cell lines with anti-p53 antibody and amplified by PCR. Primers were designed to amplify regions shown to be p53 enriched in the immunoprecipitated sample over input by ChIP-Seq data (Fig. 5Aa, b). p53 motifs (blue bars in Fig. 5Ad) identified by Genomatix and manually are shown relative to TSS (Fig. 5Ac). Species-appropriate IgG was used as negative control. PCR band intensities were quantified using the Alphaimager software as described in Methods, and band intensity of immunoprecipitated fragments was normalized to Input band intensity for each cell line. Normalized values for each amplicon (Fig. 5Ac) were plotted as mK4/mK3 ratios (Fig. 5B). Values greater than 1.0 indicate fragment enrichment in mK4 relative to mK3, as a result of increased p53 binding and immunoprecipitation as seen for amplicons P3 and P7. Ratios for rest of the fragments showed values close to 1.0, indicating p53 binding in these regions is not enhanced in mK4 cells.

p53 Activates the Pax2 Promoter in Transient Transfection Assays

In vivo gene expression and ChIP data shown above strongly suggest that Pax2 is a direct transcriptional target of p53. To further test this hypothesis, the 4.1-Pax2-reporter construct was co-transfected with a p53 expression plasmid into p33+/− H1299 human lung cancer cell-line. The 4.1-Pax2 BamHI/Not1 promoter fragment contains 3500 bp upstream to the transcription start site and ~600 bp sequences in 5′ UTR fused to the CAT or luciferase gene [51]. Greater than 15-fold increase in Pax2 promoter activity was observed in this cell-line in the presence of wild-type p53 but not with a non-DNA-binding mutant of p53, p53-E258K (Mt-p53) (Fig. 6A), indicating the requirement for p53-DNA binding for transactivation to occur. Mt-p53 acts as a dominant-negative and inhibits transcriptional activity of wild-type p53. 4.1Pax2-reporter was also responsive to p53 in two kidney cell-lines tested, one from UB-lineage (see below) and another from mesenchyme (mK4). Kidney mesenchyme-derived mK4 cells...
represent induced cap mesenchyme cells [50]. Co-transfection of pCMV-p53 expression plasmid with 4.1Pax2-Luc resulted in greater than 20-fold increase in luciferase activity compared to baseline luciferase activity from transfectants that did not receive p53 expression plasmid (Fig. 6B). Further, shRNA-mediated knockdown of p53 in mK4 cells showed corresponding decrease in endogenous Pax2 mRNA levels. p53 knock-down was achieved by transfecting mK4 cells with 4 different p53 shRNA-GFP plasmids (Fig. 6C). GFP + and - cells were separated 48 h after transfection by FACS. P53 and Pax2 mRNA levels from GFP+ cells were normalized to levels from GFP- cells. Transfection with scrambled control shRNA plasmid did not result in decreased p53 or Pax2 mRNA levels (not shown).

Collectively, our results thus far show that not only is the Pax2 promoter highly responsive to p53 but is also p53-bound both in kidneys and mK4 cells. The p53 binding motifs identified by Genomatix along the Pax2 promoter were located within or in close proximity to identified p53 peaks identified by ChIP-Seq (Fig. 6D, numbered 1–14). To test the functional relevance of the identified sites, serial truncations were made to the full-length promoter 4.1-Pax2 which has 3500 bp of DNA upstream of the TSS (Fig. 6D and E). Although the deletion constructs show increased CAT activity in response to p53, relative activity of the truncated constructs was consistently lower than from the full-length construct (Fig. 6E). The deletion constructs −3.2kbPax2-CAT and −0.45kbPax2-CAT exhibited 50% and 80% reduction in reporter activity in response to p53, respectively.

The p53 binding motifs along the Pax2 promoter (Fig. 6D, numbered 1–14) were tested for p53 binding. In EMSA, recombinant p53 showed weak binding to multiple sites (Fig. S2A). A consensus, high affinity p53 binding site [52] was used as positive control. The consensus sequence showed robust binding to p53 (Fig. S2A, complex 1). Pax2 promoter sites, however, showed weak binding. Further, several sites generated a higher mobility p53-DNA complex (complex 2), possibly from less multimerized p53 at these sites; this complex was not observed with the consensus site. To circumvent labeling efficiency issues for any probes, competition EMSA was done using p53 consensus sequence as probe and competing its binding to recombinant p53 with the identified putative sites. Binding of the p53 consensus oligoduplex to purified p53 was abolished or attenuated by addition of 200-fold excess of unlabeled p53 consensus sequence (self) or by the p53 binding site in the p21 gene (Fig. S2B, lanes 2–3). However, p53 binding sites in the Pax2 promoter competed weakly or not at all for p53 binding. Competition obtained with different sites was quantified (Fig. S2C).

To identify a specific site responsible for Pax2 transactivation, site-directed mutagenesis was done on the full-length promoter construct. Not all peaks contain consensus binding site sequences. We picked the following sites for site-directed mutagenesis because – sites 7 and 8 showed p53-binding by EMSA, sites 11 and 12 occur within the interval and peak region, and sites 12 and 14 showed high percent conservation between mammals (Table 1).

Percent conservation is the percentage of nucleotides predicted to be conserved in 30 mammalian species, calculated using the UCSC browser built-in method (Placental Mammal Basewise Conservation by PhyloP) (Methods S1). As the −3.2 kb deletion construct (without site 13) showed decreased CAT activity relative to the −3.5 kb construct (with site 13) (Fig. 6E), we also tested site 15 for functionality and response to p53 in vitro. Sites mutated by site-directed mutagenesis were re-tested by EMSA to confirm lack of binding to p53 (not shown). Point mutagenesis of single or double sites did not decrease reporter gene activity in response to p53 (Fig. 6F, and data not shown). Since multiple weak sites may promote p53 binding at the Pax2 promoter and transactivation cooperatively, further individual site mutagenesis was not pursued. In summary, our data from ChIP, p53 over-expression and promoter deletion analysis indicate that p53 binds to and transcriptionally activates the Pax2 promoter.

**Epistasis between p53 and Pax2**

In addition to regulating each other ([53] and this study), both Pax2 and p53 independently regulate a cohort of genes. To determine whether an epistatic relationship exists between the two genes, we reduced p53 gene dosage on a Pax2+/− background by crossing p53+/− to Pax2−/−/+ mice which contain the β-galactosidase gene knocked-in to the Pax2 locus. Pax2 haploinsufficiency is known to result in reduced kidney size with lower UB tip and nephron number [16]. Similarly, p53−/− kidneys exhibit renal hypoplasia with fewer UB tips [41]. Thus, we used UB tip number as a phenotypic readout of renal hypoplasia in Pax2−/−/+;p53−/− animals. On this mixed genetic background (C57BL/6; C3H/He), reduction of either Pax2 gene dosage or elimination of p53 gene reduced UB tip number by ~12-15% (Fig. 7A, B). However, complete p53−/− deficiency superimposed on Pax2 haploinsufficiency resulted in ~55% decrease in tip number, suggesting functional co-operativity between the two transcription factors.

**Discussion**

In this study we have shown that Pax2 is a physiological target for p53-mediation transcription in the embryonic kidney. Pax family of transcription factors plays a key role in lineage specification and organogenesis. Pax2 expression is essential for development of the urogenital system and mid-hindbrain pattern- ing [15,54]. While complete loss of Pax2 expression results in renal agenesis, Pax2−/− kidneys are hypoplastic. Pax2 loss in the CNS results in exencephaly [55]. p53 expression is ubiquitous early in embryogenesis. P53 activity is essential for mesodermal determination in co-operation with Smads [40,56,57]. Pax2 and p53 expression overlaps temporally and spatially in metanephric development, when expression is ubiquitous in the ureteric epithelia and surrounding metanephric mesenchyme. p53 is expressed in all cells of the embryo up to E10.5 [31], thus its expression precedes that of Pax2 in the intermediate mesoderm. However, Pax2+/− expression is required to induce and specify the nephric lineage [11]. Unlike Pax2−/− mutants, renal agenesis has never been observed or reported in p53−/− embryos suggesting a modulatory role for p53 in regulating Pax2 expression rather than an on-off regulation. Indeed, this is in line with the findings of this study, since elimination of p53 activity results in decreased Pax2 expression but not its complete loss in p53−/− kidneys.

The 4.1Pax2 promoter-reporter construct, which contains the kidney- and mid-hindbrain-specific enhancers faithfully recapitulates endogenous Pax2 expression pattern in the intermediate mesoderm and neural tissue in vivo. The transgene is expressed in the nephric duct epithelia and its derivatives but not in the metanephric mesenchyme [51]. The ability of this promoter fragment to exhibit relatively high baseline activity and response to p53 in mesenchyme mK4 cells reflect inherent differences between in vivo and in vitro models. However, decreased Pax2 expression with p53-knockdown in these cells indicates p53 does positively regulate Pax2 expression in both mesenchyme and UB cells.

Our data does not rule out the possibility of an intermediate factor X as regulator of Pax2 promoter activity. However, p53 is enriched at the Pax2 promoter in mK4 cells that express Pax2 but not in Pax2 non-expressing mK3 cells, although p53 is expressed in both cell lines. This finding, along with collective in vivo data
showing decreased Pax2 expression in germ-line p53−/− and conditional CM-p53−/− kidneys, along with in vitro transient transfection data strongly suggest p53 is directly upstream to Pax2. Unlike the deletion mutants, none of the point-mutants showed decreased activation by p53 (Fig. 6F and data not shown). Since DNA-binding activity of p53 is required to activate the Pax2

Figure 6. p53 trans-activates the Pax2 promoter. A) p53−/− H1299 cells were co-transfected with a p53 expression plasmid (pCMV-p53) and a 4.1-Pax2-CAT reporter construct. Dose-dependent increase in reporter activity was observed with wild-type p53 (WT-p53). Reporter activity was maintained at baseline when reporter plasmid was co-transfected with a non-DNA binding mutant p53 (Mt-p53, pCMV-p53-E258K). This mutant acts as a dominant-negative and inhibits Pax2 promoter activation by wild-type p53. B) mK4 cells were transiently co-transfected with a p53 expression plasmid and a 4.1-Pax2-Luc reporter construct. Absolute values of luciferase activity are plotted as Relative Light Units (RLU). Luciferase activity was normalized to protein concentration. C) p53 knock-down in mK4 cells using 4 different p53 shRNA plasmids showed corresponding decrease in Pax2 mRNA, relative to mRNA levels from GFP- cells (dotted red line). mK4 cells were transfected with p53-shRNA-GFP plasmid, and GFP+ cells were sorted 48 h post-transfection, RNA harvested and used for QPCR. Gene expression was normalized to β-actin or GAPDH expression. D) Schematic of deletion mutant Pax2 promoter constructs, shown with respect to p53 occupancy determined by ChIP-Seq. Orange bar denotes p53 occupancy in Pax2 promoter, corresponding to region 1 in Fig. 2A. Green solid arrow shows TSS used in this study, dotted arrow denotes possible alternate TSS in RefSeq. Purple bars indicate location of p53 binding sites identified in silico and tested for p53 binding by EMSA, numbered 1–14. Mutagenized sites shown in red. E) Full-length or truncated Pax2-promoter-CAT reporter plasmids were transfected into UB cells either without or with pCMV-p53 (50 ng). CAT activity was normalized to protein concentration. Fold-induction by p53 of CAT activity over baseline (red-line) is plotted. F) Fold-change in reporter activity by p53 after site-directed mutagenesis of p53-binding site 7 (Mut 7–1 and 7–2) and 8 (Mut 8–1 and 8–2) individually or together (Mut 7/8). Two clones were tested in transient transfection assays per site mutated.

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Table 1. Percent conservation in mammals of p53 binding sites in the Pax2 promoter.

| Binding site | Location on Chromosome 19 | Percent Conservation | Oligonucleotide sequence 5′→3′ |
|--------------|---------------------------|----------------------|-------------------------------|
| 1            | 44829010–44829059         | 43.75%               | cagacatac agactittaac ttaacagcc cacccagaag ggcttcatg  |
| 2            | 44829110–44829159         | 66.00%               | aagggagaga aggtctactgc ggttacttac accctagagc tctgctgcc  |
| 3            | 44829150–44829189         | 72.50%               | tctctgctcc cgtatgagc agctgacctt ttcagctgta  |
| 4            | 44828571–44828608         | 63.16%               | tttattttc cccagaaacc acgtgaaaaa ctacac  |
| 5            | 44828707–44828769         | 42.86%               | cct gggaagagc gcttgattt tcctggctga catagaaaaa ggaacttacac tctccttg  |
| 6            | 44828785–44828821         | 40.54%               | tggg cagatcttt ctactgagg cctagacag gcctgagcgc tctgagagc  |
| 7            | 44830101–44830143         | 72.09%               | ggga gcagatcggc gcagactgcag aagacaggtg ccagccgct  |
| 8            | 44830295–44830340         | 74.42%               | ggctccttcg tcctgtcggc ggcagacggc ctgctgacag tctcccc  |
| 9            | 44830465–44830518         | 59.57%               | aggggtcttgg angcatcggc ctctggggag ggggtgcgcg ccaccgcgtg  |
| 10           | 44830665–44830724         | 60.00%               | gacatctgagc caaggtgtagc gctgtagag ggcctgagc gcgtgctggg  |
| 11           | 44830735–44830794         | 73.33%               | tggccgcttg gatgcagggg ctgggctgag tagactgag agcttgctgc tggaglgccg  |
| 12           | 44831085–44831124         | 97.50%               | gatctatgct ctctgtgacg cagactacat cattcgccg  |
| 13           | 44827769–44827817         | 40.82%               | ggta gcagatcggc ttctggcttg ctagactaag cgcctggcc  |
| 14           | 44828298–44828333         | 94.44%               | aacca gcctgacgg tggagctggc gtgggtggtt  |

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Pax2 positively regulates expression of pro-survival gene Bcl2 [71] whereas p53 is a known transactivator of pro-apoptotic genes such as Bax, PUMA and Noxa [67, 72, 73]. Pax2 deficiency results in increased apoptosis [17, 74]. Although this study did not compare the apoptotic index between wild-type, single and Pax2Δ2–2, p53Δ2–2 double mutants, the worsening hypoplasia upon p53 deletion in Pax2Δ2–2/+ kidneys suggests increased apoptosis may result from further Pax2 decrease as a result of p53 loss. Effects of p53 deletion are genetic background dependent, Pax2Δ2–2/+ mice are on a C57/16 background [13]. When p53Δ2–2/mice are crossed to this background, these mixed background mice did not show as severe a renal phenotype as we observed in C57BL/6 mice.

Given that Pax2 is a proto-oncogene, promotes cell survival and is up-regulated in Wilms’ tumour and renal cell carcinoma [10, 75], why does p53, a tumor suppressor, positively regulate Pax2 expression? In addition to lineage specification and promoting cell survival, Pax2 is also implicated in promoting mesenchyme-to-epithelial transition and cell differentiation [76]. We propose a feedback model wherein Pax2 in the nephrogenic zone down-regulates p53 expression [53] in the self-renewing cap mesenchyme (Fig. 8B). Our data from conditional p53Δ2–2 deletion kidneys (CM Pax2Δ2–2/+ ) show recapitulation of renal hypoplasia observed in germ-line p53Δ2–2/+ kidneys. CM p53Δ2–2/+ kidneys exhibit decreased Pax2 levels specifically in the CM (Fig. 3A) indicating that p53 levels in the CM are sufficient to enhance Pax2 expression in mesenchyme cells and promote their transition to epithelia. An alternate explanation for decreased Pax2 staining in the CM may be precocious differentiation of CM to nascent nephrons. To address this possibility, we stained embryonic and post-natal kidneys sections with Lhx1, a marker for nascent nephrons. Not only is Lhx1 staining decreased in the mutant kidneys (Fig. 1D), but these kidneys showed no ectopic Lhx1+ differentiated structures that would be expected if these cells were undergoing precocious differentiation. Modulation of p53 function by Pax2 may be viewed as a cell survival mechanism, since unconstrained p53 activity results in excessive apoptosis [46].

We conclude that p53 serves to promote differentiation of progenitor cells to nascent nephrons, and in its absence Pax2 down-regulation is a possible mechanism of renal hypoplasia observed in these kidneys. We propose that the cross-talk between...
p53 and Pax2 provides a transcriptional platform that promotes nephrogenesis, thus contributing to nephron endowment. Our data contribute to further elucidate mechanisms regulating de novo nephrogenesis in the embryonic kidney, an area of interest not only for potential use in in utero intervention but also from the point of regenerative medicine.

Materials and Methods

Mice

p53<sup>+/−</sup> mice on C57BL/6 background (Jackson labs) were time-paired to obtain E15.5 p53<sup>+/−</sup> and p53<sup>−/−</sup> litter-matched kidneys. For conditional p53 deletion in cap mesenchyme, Six2<sup>GC</sup>:p53<sup>loxP+/+</sup> mice were crossed to p53<sup>loxP+/loxP</sup> mice and embryonic kidneys obtained from resultant crosses. The Six2<sup>GC</sup> mice were a gift from A. McMahon [3]. Pax2<sup>LacZ+/+</sup> knock-in mice were a kind gift of M. Bouchard, and maintained on a C3H/He background [13]. For epistasis experiments, Pax2<sup>LacZ+/+</sup>:p53<sup>+/−</sup> mice were generated and crossed to p53<sup>−/−</sup> to obtain p53<sup>−/−</sup> mice on a Pax2<sup>+/+</sup> background. All animal protocols utilized in this study were approved by and in strict adherence to guidelines established by the Tulane University Institutional Animal Care and Use Committee.

Immunofluorescence Staining

E15.5 kidneys were fixed in 10% formalin and processed for paraffin embedding and sectioning. Five micrometer paraffin sections were subjected to antigen retrieval (10 mM sodium citrate, pH 6.0) after deparaffinization and rehydration. Immunostaining was done as previously described [47]. Antibodies for the...
following proteins were used: p53FL (SantaCruz, 1:500) and Pax2 (1:200, Zymed/Invitrogen). DAPI (1:500, D1306, Invitrogen) was used to stain nuclei. Whole-mount staining was done on E12.5–13.5 kidneys as previously described [41] with Pan-cytokeratin antibody (Sigma, 1:200). The immunofluorescent images were captured using a 3D or deconvolution scope (Leica DMRXA2) and staining intensity was quantified using Intelligent Imaging Innovations SlideBook software.

Chromatin Immunoprecipitation (ChIP) Assays

Chromatin was prepared from 15 pairs of E15.5 kidneys. After shearing, p53-bound chromatin was immunoprecipitated with p53FL antibody (SantaCruz) and subjected to ChIP coupled to
Table 2. ChIP-PCR primer sequences.

| Primer | Chr.19: Amplicon Start | Chr.19: Amplicon End | Amplicon (bp) | Sequence |
|--------|------------------------|----------------------|--------------|----------|
| P1     | 44830940               | 44831180             | 240          | F: atctcaacctctcccttcttc R: tggcgctctatccatatgtc |
| P2     | 44830233               | 44830617             | 384          | F: tctccagctcttctcttcttc R: gctctcccccttgcctt |
| P3     | 44829920               | 44830314             | 394          | F: gctgggagggagggagaa R: gcagatctacgagagggag |
| P4     | 44829512               | 44829938             | 426          | F: gttctacatccagagag |
| P5     | 44829275               | 44829678             | 403          | F: tttctatgagaagttagggtgt R: ctctgtaccccttgcacag |
| P6     | 44828895               | 44829328             | 433          | F: tatccatgtctttgcaaca R: caatctccatcactcaca |
| P7     | 44828544               | 44828972             | 428          | F: ctctcaacagcagagggtag R: gtctctccatccagag |

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next generation sequencing (ChIP-Seq) using the Illumina protocol on the Solexa GAII. p53 peaks were called using the MACS program [77]. p53 antibody (Santa Cruz, SC-6243x) was used for all p53 ChIP experiments. ChIP-PCR on mK3 and mK4 cells was performed using reagents and protocols from the Upstate Biotechnology ChIP kit with modifications as previously described [78]. PCR primer sequences are shown in Table 2. ChIP tracks showing p53 occupancy at the gene locus were generated by Integrated Genome Browser (IGR http://bioviz.org/igb) using ChIP-Seq-derived sequence files.

In situ hybridization
Whole mount in situ hybridization was performed as described [79]. E10.5-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 overnight. Embryonic tissue was photographed in glycerol.

p53 knockdown and RT-qPCR
mK4 cells were transfected with p53-shRNA-GFP plasmid (SuperArray Biosciences, KM0931G) for 72 h. Transfected cells were sorted by FACS and RNA was isolated from GFP+ and GFP− cells. Real-time primers for p53 (TaqMan Gene Expression Assay, ID: Mm01217939_m1) and Pax2 (TaqMan Gene Expression Assay, Mm01217939_m1) were from Applied Biosystems. The TaqMan expression assay was constituted using reagents from the TaqMan QRT–PCR 1-step Master Mix kit (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. Expression was normalized to GAPDH or β-actin levels.

Plasmids
4.1Pax2-Luc, 4.3Pax2-CAT and truncated Pax2-CAT constructs were a kind gift of G. Dressler [51]. The p53 expression vectors have been described previously [80]. Site-directed mutagenesis was done using the QuickChangeTM Site-directed Mutagenesis System (Stratagene, La Jolla, CA) as per manufacturer’s directions.

Cell Culture, Transient Transfection, and Reporter Assays
p53-null human lung carcinoma cells H1299 (ATCC), an E11.5 mouse ureteric bud cell line (UB cells; kind gift of J. Barasch) and mouse kidney cap mesenchyme cell lines (mK3 and mK4 cells; kind gift of S. Potter) were maintained in media supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator with 5% CO2. Cells were transfected with 1.0 μg of DNA/well promoter-reporter vectors along with pCMV-p53-(wt) or pCMV-p53-(mutant) expression plasmids (0–250 ng). Transfection was performed using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s recommendations. Four hours after transfection, fresh medium was replaced, and cell extracts were prepared 24–48 h later using a reporter lysis reagent (Promega). Aliquots of cell lysate were analyzed for CAT or luciferase activity after normalization for protein content or β-galactosidase activity as previously described [81].

Supporting Information
Figure S1  A) p53 is expressed in both mK3 and mK4 cells. Western blot was done on whole cell lysates, and B) Pax2 is expressed in mK4 cells but not in mK3 cells. (TIF)

Figure S2 EMSA shows p53 binding to in silico identified sites in and around the p53-enriched region in the Pax2 promoter. A) 32P-labelled oligonucleotides (Table 1) were incubated with recombinant, purified C terminus-truncated and constitutively active p53. C, p53 consensus sequence ( [52]). Free probe and DNA-p53 complexes are indicated. B) Competition by p53 binding sites identified in Pax2 promoter with the p53 consensus sequence. Labeled consensus oligoduplex was incubated with p53 alone (lane 1) or in presence of unlabelled competitor oligoduplexes as described in Methods S1. Diminished consensus-p53 complex indicates effective competition. C) Inhibition of complex formation between p53 and consensus binding site by
addition of various unlabelled competitor oligoduplexes was quantified and plotted. Unlabelled consensus binding site and p21 promoter-p53 binding site compete effectively for p53 binding, whereas p53 binding sites from the Pax2 gene region (sites 1–14) show weak competition.

(TIF)

Figure S3 p53 binding sites are broadly scattered across the entire region including the intervening region between regions 1 and 2. IGB view of Chromosome 19:44,830,572 - 44,836,404 is shown. a) Location of p53 binding motifs identified by Genomatix; b) Vertical green bars show p53 binding sites in p53-occupied region denoted by horizontal green bar; c) p53 Chip-track; d) Input track; e) Mus Chromosome 19 coordinates.

(TIF)

Methods S1 Supporting methods file.

(DOCX)

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