Hereditary renal cystic diseases are characterized by defects in primary cilia of renal tubular epithelial cells and abnormality of tubular epithelium, which ultimately result in the development of renal cysts. However, the mechanism leading from abnormality of the tubular epithelium to cystogenesis is not well understood. In this report, we demonstrate a critical role for Robo2 in regulating epithelial development, including ciliogenesis, polarization, and differentiation. We found that Robo2 deficiency results in cystic kidneys, and the cyst cells showed defective cilia and polarity defects in tubular epithelium. The cyst cells, less than terminally differentiated, continue to proliferate. We further established that Robo2 works with p53 as well as polarity and ciliary proteins (Par3, PKCζ, ZO-2, and Claudin-2) to regulate these processes. Robo2 binds to Baiap2 (also known as IRSp53) through the IRSp53/MIM homology domain in renal epithelial cells. This binding allows Robo2 to phosphorylate MDM2 at Ser166 via Baiap2 and maintain p53 homeostasis. Disruption of the Robo2-Baiap2 complex causes MDM2 to be subjected to dephosphorylation, leading to a high level of active p53, and initiated p53-mediated cellular senescence via p21 and decreased the expression of ZO-1, ZO-2, PKCζ, Par3, and Claudin-2 proteins, resulting in defects in epithelial development, including ciliogenesis, polarization, and differentiation. Importantly, double knockout of Robo2 and p53 rescued all the epithelial defects in kidneys compared with those […]
Disruption of Robo2-Baiap2 integrated signaling drives cystic disease

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Introduction

Renal cystic diseases include autosomal dominant polycystic kidney disease, autosomal recessive PKD, and others (1, 2). Moreover, most cysts are induced by the disruption of primary cilia and polarity (1, 2). Numerous kidney cysts accompany these polarity defects (3). Aberrant polarization of tubule cells results in inappropriate location of Na⁺/K⁺ channels to the apical domain of the cells, resulting in dilation of tubule lumens. The proper polarization and differentiation of renal tubule epithelial cells are essential for kidney development that prevents abnormal cystic dilation.

The fundamental process of cellular differentiation polarization is established early during development (4). Multiple proteins and pathways have been identified as regulators of epithelial development throughout the polarization process. The apical-basal polarity of cells is a prerequisite for all aspects of epithelial cell function and is required for the proper localization of primary cilia (5), and cilia are required for maintaining cell polarity (5). The primary cilia of renal tubular epithelial cells also function to maintain normal tubular architecture, and dysfunction of primary cilia alters renal tubular cell proliferation and differentiation. However, interplay between the many pathways for epithelial development and function is still unclear.

Robo2, the cell surface receptor for the repulsive guidance cue Slit, is involved in axon guidance and neuronal migration (6, 7). The importance of Robo2 signaling in metanephric kidney development...
and renal tubular formation is underlined by the knockout mouse model for Robo2 as well as heterozygous ROBO2 mutations in humans with congenital anomalies of the kidney and urinary tract \( (8–12) \). We found that Robo2 deficiency results in cystic kidneys, and the cyst cells in the kidneys showed defective cilia and polarity defects in tubular epithelium. The link between abnormality of the tubular epithelium and cystogenesis remains elusive. We hypothesized that Robo2 plays a critical role in regulating epithelial development, including ciliogenesis, polarization, and differentiation. To address these unanswered questions, mouse models with selective deletion of Robo2 or p53 alleles were used for the following investigations.

**Figure 1. Robo2 is required for progenitor cells to differentiate into ciliated and polarized epithelium.** (A) Representative H&E-stained image showing renal cysts (*) in the Robo2\(^{-/-}\) mice. Scale bars: 50 \( \mu \)m. (B) Immunofluorescence (IF) staining of Robo2 and Pax8 in renal tubular epithelial cells in E15.5 wild-type kidneys \((n = 5)\). Scale bars: 50 \( \mu \)m. (C) IF staining of Pax8 and Lotus tetragonolobus lectin (LTL) in renal tubular epithelial cells in E17 wild-type and Robo2-knockout kidneys. Pax8 was expressed in cystic segments with reduced or absent LTL staining (white arrow and arrowhead). The representative image shows cysts (*) in the kidney. Scale bars: 50 \( \mu \)m. (D) IF staining of Aqp1 and LTL in the cryosections of E17 wild-type and Robo2\(^{-/-}\) kidneys. The misexpression of LTL and Aqp1 was found in the Robo2\(^{-/-}\) kidneys. Scale bars: 10 \( \mu \)m. Biological replicates: \( n = 10 \). (E) IF staining of \( \alpha \)-tubulin and LTL in the cryosections of kidneys showed the defects of ciliogenesis in the Robo2\(^{-/-}\) kidneys \((n = 7)\). Scale bars: 10 \( \mu \)m. (G) Transmission electron micrographs show that primary cilia (white arrow) defects (white arrowhead) occurred in the Robo2\(^{-/-}\) kidney. Scale bars: 200 \( \mu \)m. (H) The tight junction (white arrowhead) is disrupted in the Robo2\(^{-/-}\) kidney. Scale bars: 500 \( \mu \)m.
Results

Robo2 is required for progenitors to differentiate into renal tubular epithelial cells. Knockout of Robo2 resulted in defects in the Robo2−/− kidneys (Figure 1A). To investigate whether Robo2 is involved in epithelial differentiation, we stained the E17 wild-type kidney sections and found that Robo2 was located in embryonic renal tubular cells (Figure 1B, left) and coexpressed with Pax8 (13–15) (an early proximal tubule marker) in these cells (Figure 1B). We further found that the abnormal epithelial cells from Robo2−/− kidneys stained positive for Pax8 (Figure 1C), Cited1 (a nephron progenitor marker) (refs. 16, 17; and Supplemental Figure 1B; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.127602DS1), Sox9 (Sox9+ cells are progenitors in the kidney) (refs. 18–21 and Supplemental Figure 1C), and Aqp1 (a proximal tubule apical marker) (Figure 1D), whereas the staining of LTL (a proximal tubule apical marker) in these cells was weak, undetectable, or mislocalized (Figure 1, C–E, and G) compared with that in wild-type kidneys (22). In addition, the staining of Na+/K+-ATPase, a cell polarity marker, was markedly decreased in the Robo2−/− kidney compared with that in the wild-type kidneys (Figure 1E). The mislocalization of LTL and Na+/K+-ATPase on the apical membranes suggested a lack of polarization and terminal differentiation in cyst lining epithelial cells in Robo2−/− kidneys. We further examined the embryonic stage including E15.5 of mutant kidneys to show that failures in differentiation of renal progenitor cells into renal tubules occur from the beginning of nephrogenesis (Supplemental Figure 1A).

We also found that cilia were detected by the staining of α-acetylated tubulin, a cilia marker, in tubular cells that were costained with LTL in wild-type kidneys (Figure 1F). However, knockout of Robo2 disrupted ciliogenesis (Figure 1G) and tight junction (Figure 1H) and resulted in short and dot-like cilia in cyst lining epithelial cells (Figure 1, F and G). We further found that knockout of Robo2 also resulted in short and dot-like cilia in mouse inner medullary collecting duct (IMCD3) cells (Supplemental Figure 2, A–C). These results suggested that the Robo2-deficient renal epithelial cells were incompletely differentiated and had defects in differentiation, including cell polarity and ciliogenesis.

Binding of Robo2 to Baiap2 forms a functional signaling complex. To understand the mechanism of how Robo2 regulates epithelial cell development/differentiation, polarization, and ciliogenesis, we identified the in vivo binding partners of Robo2 using coimmunoprecipitation (co-IP) (Supplemental Figure 3A) and mass spectrometric analysis (Supplemental Table 1). We found that Baiap2 (also known as Isp53) might be the most likely candidate protein for Robo2 interaction.

We found that Baiap2 was colocalized with Robo2 and LTL basolaterally in embryonic epithelial cells in wild-type kidneys (Supplemental Figure 4, A and B). We confirmed the interactions between Robo2 and Baiap2 with His- and Myc-tagged human full-length Robo2 (His-myc-Robo2) or Flag-Baiap2 in HEK293T cells by co-IP assays (Figure 2A). Because Robo2's biological function depends upon the cc domain (Figure 2B and ref. 23), we made several His- and Myc-tagged constructs of Robo2 with different cc domains and found that the crucial binding site of Robo2 with Baiap2 was located within amino acids 1301–1394 (CC3-1394, amino acids 1301–1394, which span from CC3 domain [amino acid 1301] to amino acid 1394) (Figure 2, C and D, and ref. 23). Last, we determined that Robo2 interacted with Baiap2 through the IMD (amino acids 1–250) but not the SH3 domain (amino acids 251–521) (Figure 2, D and E, and refs. 24–26) because His-myc-Robo2 (CC3–1394) could be co-IP only with Flag-Baiap2-IMD but not with Flag-Baiap2-SH3 with Myc beads (Figure 2, D, E, and G). An in vivo co-IP experiment using kidney tissue lysates also showed that Robo2 interacted with Baiap2 in vivo (Figure 2F).

To rule out an indirect association of Robo2 and Baiap2 in a protein complex and demonstrate the direct interaction of Robo2 and Baiap2, we did a pull-down assay to prove the direct interaction of Robo2 and Baiap2 (Supplemental Figure 3, B–E).

To explore the functional role of the interaction between Robo2 and Baiap2, we coexpressed these proteins in Madin-Darby canine kidney (MDCK) cells. We found that Robo2 could recruit Baiap2 to the cell membrane in cells coexpressing Robo2 and Baiap2 (Supplemental Figure 4C), and 68% of cells were positive for both Robo2-GFP and Flag-Baiap2. In contrast, the cells transfected with Baiap2 alone showed a diffuse punctate distribution of Baiap2 throughout the cytoplasm rather than membrane localization (Supplemental Figure 4C). Similar to the localization pattern of Baiap2 in vitro, we found that Baiap2 was accumulated at the tight junctions in the wild-type mouse kidneys and was almost undetectable in the Robo2−/− kidney (Supplemental Figure 4B). Previous reports demonstrated that Baiap2 is associated with epithelial differentiation and polarization (27), our results suggest that the localization of Baiap2 depends on the presence of Robo2, which may be required for cell differentiation.
Figure 2. Robo2 directly interacts with Baiap2 via its IRSp53 and MIM domain. (A) Co-IP of Robo2 and Flag-tagged Baiap2 using the anti-Flag antibody. (B) Schematic diagram of the domain structure of the Robo2 and Baiap2 proteins. Ig, immunoglobulin domain; FN III, fibronectin type 3 domain; CC, cytoplasmic region; IMD, IRSp53/MIM homology domain (amino acids 1–250); SH3, Src homology 3 domain; WH2, WASP-homology 2 domain. The numbers indicate the positions in the full-length Robo2 or Baiap2 gene. (C) The co-IP assays showed that the region from CC3-1394 in Robo2 is required for its interaction with Baiap2. (D and E) The co-IP assays showed that the IMD of Baiap2 is required for its interaction with the region from CC3-1394 in Robo2. (F) Co-IP analysis of endogenous Robo2 interacting with the exogenous Baiap2 using kidney tissue lysates. (G) The schematic diagram shows the binding domains of Robo2 and Baiap2.
Robo2 also suggested the association of ZO-1 with the differentiation defects in the Tjp1, in these mice (Figure 3B). Knockdown of Trp53 genes, including proteins and the downregulation of differentiation genes. We selected and confirmed the expression of genes controlling cell cycle and cellular adhesion were also involved in the upregulation of p53 signaling proteins and the downregulation of differentiation genes. We selected and confirmed the expression of 9 genes, including Trp53 (p53), Cdkn1a (p21), Tjp1 (ZO-1), Cdkn1b, B-Raf, Cdc42, Ctnna1, Ctnnb1, and Pten by NanoString nCounter Gene Expression Assay (Figure 3B), which play important roles in mediating senescence and differentiation (28–32). The expression of p53 and p21 proteins was increased, and the expression of cyclin-dependent kinase 4 (Cdk4) protein was decreased in Robo2−/− kidneys compared with that in wild-type kidneys in vivo (Figure 3C).

We further found that knockdown of Robo2 not only increased the nuclear p53 in MDCK cells (Figure 4A) but also led to DNA damage in these cells as shown by γ-H2AX staining (a hallmark of DNA damage) (Figure 4B and ref. 33). Knockdown of Robo2 or Baiap2 also showed a high level of γ-H2AX by Western blot (Figure 4C). Strikingly, the γ-H2AX+ or p53+ cells displayed large, flattened, senescence-like nuclei (Figure 4, A and B). Additionally, senescence-associated β-galactosidase (SAβ-Gal) activity was increased in the renal tubules (Figure 4D) from Robo2−/− mice, with the exception of the metanephric mesenchyme, which is not observed in other models (34). Consistent with the elevated p53 and p21 levels, the Robo2−/− mice exhibited p21 expression in epithelial cells lacking differentiation and polarization (Supplemental Figure 5B). Taken together, these data suggest that loss of Robo2 could induce senescence, which might be through the unreported p53/p21 pathway.

Because loss of Robo2 led to the upregulation of p53 and resulted in p53-mediated senescence (Figure 3, A–C; Supplemental Figure 6B; and ref. 29), and knockdown of Robo2 or Baiap2 also showed high level of γ-H2AX by Western blot (Figure 4C), we investigated the involvement of the Baiap2 cascade in p53 signaling. It has been reported that the phosphorylation of MDM2 promotes p53 degradation while the dephosphorylation of MDM2 can stabilize nuclear p53 (35, 36). We found that Robo2 was required to maintain the phosphorylation of MDM2 at Ser166 because Robo2 deficiency led to the dephosphorylation of MDM2 in vitro and in vivo (Figure 4, E and F, and Supplemental Figure 6, A and B). We further found that Baiap2 deficiency also caused dephosphorylation of MDM2 at Ser166 (Figure 4G). These data indicate that Robo2 deficiency results in the dephosphorylation of MDM2 and subsequent phosphorylation of p53 through the Baiap2 cascade, ultimately resulting in the upregulation of p53. Further, knockdown of Robo2 or Baiap2 alone also resulted in upregulated expression of H2AX in MDCK cells (Figure 4C), which suggested that silence of Robo2/Baiap2 can contribute to DNA damage and even induce cellular senescence.

We further found that Slit2 has no significant direct effect on Baiap2, p53, and phosphorylated MDM2 after Robo2 knockdown (Supplemental Figure 6A), although Robo2 is the receptor for repulsive ligand Slit2. These data suggested that the effects of the Robo2-Baiap2 signaling pathway seem independent of Slit regulation on the Robo2 receptor.

Silence of Robo2 disrupts the normal program of cellular differentiation. Because ZO-1 is required for epithelial morphogenesis and lumen development (37–39), we further found that the expression of ZO-1 in renal epithelial cells disappeared in cyst lining epithelial cells in the Robo2−/− kidney (Figure 5A), which was consistent with the downregulation of the ZO-1 encoding gene, Tjp1, in these mice (Figure 3B). Knockdown of Robo2 also dramatically decreased the expression of ZO-1 in MDCK cells (Figure 5, B–D). These results also suggested the association of ZO-1 with the differentiation defects in the Robo2−/− kidneys.

We further found that silence of Robo2 not only decreased the expression of ZO-1 in vivo and in vitro (Figure 3B, Figure 5, A–D, and Figure 6A) but also decreased the in vivo expression of PKCζ, Par3, Claudin-2, and ZO-2 (Figure 5E), which are members of the ciliary and polarity protein complex (37, 40, 41). The expression of ZO-1 could be found in Robo2−/− kidneys (Figure 6A and Supplemental Figure 6B). Collectively, our results suggest a requirement for the Robo2 signaling cascade in progenitor differentiation. Moreover, because PKCζ, Par3, and Claudin-2 are also ciliary proteins (5, 41), it is possible that these proteins might also be involved in the ciliogenesis defects found in the Robo2−/− kidneys (Figure 1, F and G, and Supplemental Figure 2, A–C).
Robo2 mutations result in the abnormality of tubular epithelium because of p53-mediated signaling. To investigate the relationship between cell differentiation and p53-mediated senescence and determine whether the phenotypes in Robo2−/− kidneys can be rescued by suppressing p53-mediated senescence, we generated Robo2−/− p53−/− double knockout mice. We found that double knockout of Robo2 and p53 restored the expression of Cdkn1a (p21) as examined by the NanoString assay analysis (Figure 3B) as well as the expression of P21, CDK4, PKCζ, Claudin-2, and ZO-2 in kidneys compared with those in wild-type kidneys. Data are mean ± SEM. **P < 0.01 by 2-tailed Student’s t test.

Figure 3. Knockout of Robo2 changes the transcription of genes associated with different signaling pathways in Robo2−/− kidneys compared with those in wild-type kidneys. (A) The gene expression profiles between the E17 wild-type and Robo2−/− mouse kidneys. The double-headed arrows indicate that 240 genes were upregulated and 232 genes were downregulated by Robo2. Some of the Robo2-repressed genes (black) and Robo2-induced genes (green) are listed vertically (left), and some of the potential Robo2-related molecular pathways are indicated on the right. (B) NanoString nCounter Gene Expression Assay was used to confirm the changes of the transcription of the indicated genes identified in the microarray analysis. The RNA was purified from E17.5 Robo2−/−, Robo2−/− P53−/−, P53−/−, and wild-type kidneys. The relative expression is shown as the fold difference relative to Gapdh, Actb, and B2m. (C) The expression of p53 and p21 proteins was increased, and the expression of CDK4 proteins was decreased in the Robo2−/− kidneys compared with those in wild-type kidneys. Data are mean ± SEM. **P < 0.01 by 2-tailed Student’s t test.
Moreover, we found that double knockout of Robo2 and p53 prevented renal cyst formation (Figure 6, B, C, and F) and normalized the cell polarization defects compared with those in the Robo2–/– mouse kidneys (Figure 6D). In addition, the SAβ-Gal staining in the renal tubule cells was absent in the Robo2–/–p53–/– double homozygous mouse kidneys in comparison with the Robo2–/– mouse kidneys (Figure 6, E and G). These findings indicate that Robo2 mutations result in differentiation defects because of p53-mediated progenitor cell senescence (Figure 7).

Discussion

Renal cystic diseases encompass a wide spectrum of inherited, developmental, and acquired disorders (1, 2), and the hereditary renal cystic diseases are caused by abnormality of tubular epithelium, including defects in differentiation, polarization, and ciliogenesis. In this study, we found that Robo2 deficiency results in cystic kidneys and described the potentially novel roles and mechanisms of Robo2 in regulating renal tubular epithelial cell development. We found that deletion of Robo2 induced renal epithelial defects on progenitor differentiation, polarization, and ciliogenesis in vivo. We successfully showed that Robo2 regulates these processes through Baiap2-mediated p53 as well as ciliary and polarity proteins Par3, PKCζ, ZO-2, and Claudin-2.

Baiap2 is a multidomain protein that is able to associate with the Rho-GTPases. Rac is required for epithelial differentiation, mainly through its amino-terminal IMD (27, 42). We found that Baiap2 bound to Robo2 via this IMD (Figure 2G), which suggested that these proteins could form a protein complex. Our data indicated that total p53 protein can be upregulated after Robo2 knockout (Figure 3C and Supplemental Figure 6B) or Robo2 siRNA knockdown (Supplemental Figure 6A), although the phosphorylation of p53 was not affected (Figure 4F). MDM2 is well known as an ubiquitin E3 ligase for p53; therefore, it acts to degrade p53. The regulation of MDM2 is mediated by its interaction with p53, leading to p53 ubiquitination and degradation (43). The stability of MDM2 can be regulated by phosphorylation at Ser166, making it a crucial regulatory residue. Akt-mediated phosphorylation of MDM2 at Ser166 results in MDM2-mediated ubiquitination and degradation of p53 (44, 45). Additionally, MDM2 has a role in abrogating the activity of p53 to induce growth arrest and apoptosis. Importantly, the interaction between p53 and MDM2 can be modulated at sites on both molecules, so the regulation of MDM2 is equally important in the p53 responses. We found that Robo2 and Baiap2 were required for maintaining the phosphorylation of MDM2 at Ser166 because Robo2 or Baiap2 deficiency led to the dephosphorylation of MDM2 and the upregulation of p53 (Figure 4, F and G; Supplemental Figure 6, A and B; and Figure 7). Abnormally high levels of wild-type p53 in the ureteric bud appear to alter cellular differentiation in embryonic kidney and cause insufficient conversion to epithelium in the adjacent undifferentiated mesenchyme (46). The UBmD2–/– mutant phenotype is mediated by aberrant p53 activity (47), which is similar to the Robo2-deficient phenotype in our study. Our previous experiment on cysts derived from Pkd1-mutant renal epithelial cells showed deacetylation of p53 (48), which indicated that the level of p53 was crucial for renal cyst formation. Thus, our results provide the first demonstration to our knowledge of an important role played by Robo2 in the modulation of p53 activity in renal tubules and stability via regulating the phosphorylation of MDM2, which further supports that MDM2-mediated inhibition of p53 activity is a prerequisite for renal organogenesis.

Multiple observations led to the hypothesis that the senescent cells do not reach terminal differentiation. Similar to the depolarized and dedifferentiated epithelial cells in cystic renal disease, the Robo2-deficient epithelial cells also displayed arrested differentiation and polarization. We found that the Robo2–/– epithelial cells positive for Pax8 (Figure 2B), Cited1 (Supplemental Figure 1B), Sox9 (Supplemental Figure 1C), and E-cadherin staining (Supplemental Figure 5A) remained as progenitor cells. These results suggest that the Robo2–/– tubule epithelial cells had not yet undergone terminal differentiation. Robo2 colocalized with tight junction protein ZO-1 and Claudin-2 (Supplemental Figure 4, D and E), and works together with polarity proteins Par3, Par6, and PKCζ (37, 40, 41) as well, which are required for epithelial polarization (49). In this study, we found that Robo2 deficiency results
in decreased expression of ZO-1, Claudin-2, and Par3/Par6/PKCζ, finally contributing to abnormal tubule epithelial cell polarity in the mutants. These results demonstrate that Robo2, as a potentially novel tight junction protein, plays an essential role in the establishment and maintenance of apical-basal polarity during epithelial morphogenesis and lumen development.

The differentiation of tubular lining epithelial cells is essential for normal kidney development, and membrane protein polarity defects include the misplacement of normally basolateral membrane proteins to the apical surface and the failure to traffic and insert proteins into membranes, resulting in their intracellular accumulation. We found that polarity proteins Claudin-2 and Par3 were decreased in Robo2−/− kidneys and in Robo2-knockdown MDCK cells (Figure 5E). Par3 is required for the establishment of epithelial cell polarity (49). Par3 inactivation results in defective convergent extension and tubular morphogenesis as well as renal cyst formation (40). Claudin-2 is present at the cilium as a ciliary protein (41), which is also crucial to epithelial cell polarization (37, 50). The apical-basal polarity is also required for the proper localization of cilia (5). Our results suggest that Robo2-Baiap2 signaling may be required to recruit polarity and ciliary proteins, including Par3 and Claudin-2, to regulate epithelial differentiation, including polarization and ciliogenesis.

We further hypothesized that the abnormality of tubular epithelium was due to the abnormal activation of p53 (Figure 7). Appropriately, we found that a double knockout of Robo2 and p53 not only rescued

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**Figure 5. Silence of Robo2 signaling disrupts the normal program of cellular differentiation.** (A) ZO-1 disappeared from cyst-lining epithelial cells in the Robo2−/− kidneys. Scale bars: 10 μm. (B) Knockdown of Robo2 decreased ZO-1 expression in the MDCK cells and induced senescence-like cells (arrow). The red dots also show the labeling of siRNA with Cy3 (Robo2 siRNA), but the control siRNA was not labeled. Scale bars: 10 μm. (C) IF staining of γ-H2AX and ZO-1 in Robo2-knockdown MDCK cells. Knockdown of Robo2 decreased ZO-1 expression in the MDCK cells and induced senescence (arrow). The red dots just show the labeling of siRNA with Cy3 (Robo2 siRNA). (D) Robo2 knockdown significantly decreased the ZO-1 protein level in the MDCK cells. Data are mean ± SEM. **P < 0.01 by 2-tailed Student’s t test. (E) The expression of the polarity proteins, including ZO-2, PKCζ, Par3, and Claudin-2, was decreased in Robo2−/− kidneys compared with that in wild-type kidneys (n = 5). Data are mean ± SEM. **P < 0.01 by 2-tailed Student’s t test.
Figure 6. Double knockout of Robo2 and p53 rescues the defects observed in Robo2-single knockout kidneys. (A) Double knockout of Robo2 and p53 rescued expression of p21, ZO-1, ZO-2, PKCζ, and Claudin-2 (n = 3). Data are mean ± SEM. *P < 0.05; **P < 0.01 compared with wild-type group, 1-way ANOVA with Bonferroni’s post hoc test. (B) Double knockout of Robo2 and p53 rescued the Robo2-deficient kidney phenotype. No renal cysts could be found in the Robo2-/-p53-/- kidney. White arrows indicate cysts. (C and F) Double knockout of Robo2 and p53 prevented cyst formation (see asterisks), which was observed in Robo2-deficient kidneys, and histological analysis revealed that the morphology of the renal tubules in the double homozygous mice appeared relatively normal. Cystic index in Robo2-/- versus Robo2-/-p53-/- kidneys shows a significant reduction in the number of cysts in Robo2-/-p53-/- animals. Data are mean ± SEM. *P < 0.05, **P < 0.01 compared with wild-type group, 1-way ANOVA with Bonferroni’s post hoc test (F). Scale bars: 10 μm (C). (D) The cell differentiation defects in Robo2-/- kidneys were normalized in Robo2-/-p53-/- double knockout kidneys. Scale bars: 10 μm. (E and G) SAβ-Gal staining indicated that double knockout of Robo2 and p53 decreased senescence in Robo2-/-p53-/- kidneys compared with that in Robo2-/- kidneys and relative quantification. Asterisks in E indicate cysts. Data are mean ± SEM. **P < 0.01 compared with wild-type group, 1-way ANOVA with Bonferroni’s post hoc test (G). Scale bars: 20 μm (E). (n = 3).
the expression of PKCζ, ZO-2, and Claudin-2 but also p21, CDK4, and SAβ-Gal. These results suggest that Robo2 regulates epithelial development by modulating Baiap2 signaling to p53 (Figure 7).

In summary, the present study provides evidence that Robo2-Baiap2 integrated signaling plays a crucial role in tubular epithelial cell development, including ciliogenesis, polarization, and differentiation of tubular epithelium. Robo2 deficiency caused cystic kidneys by defects of ciliogenesis, polarization, and differentiation of tubular epithelium. This conclusion is in line with observations in patients with renal cystic diseases (1, 2) and suggests a clinical opportunity to explore renal cystogenesis to target cystic disease.

**Methods**

*Mice.* B6.129S2-Trp53<sup>tm1Tyj</sup>/J (stock 002101) mice were purchased from The Jackson Laboratory. B6.129P2-Robo2<sup>tm1Mrt</sup>/Mmmh (MMRRC: 030409-MU) mice were purchased from MMRRC/University of Missouri.

**Antibodies.** The primary antibodies included anti-Robo2 (catalog sc-31607, Santa Cruz Biotechnology), anti-Robo2 (catalog MAB3147, R&D Systems), fluorescein-conjugated LTL (catalog FL1321, Vector Labs), anti–phospho-histone H3 (Ser10) (catalog 9701S, Cell Signaling Technologies), rhodamine-conjugated phalloidin (R415, Invitrogen), anti–myc (9B11) mouse mAb (Sepharose Bead Conjugate, catalog 3400, Cell Signaling Technologies), anti–E-cadherin (catalog AF748, R&D Systems), anti-Baiap2 (catalog 11087-2-AP, Proteintech), anti–E-cadherin (catalog ab53033, Abcam), anti–ZO-1 (catalog 617300, Invitrogen Zymed), anti-Myc (71D10) (catalog 2278, Cell Signaling Technologies), antim laminin (catalog L9393, MilliporeSigma), anti–PKCζ (catalog sc-216, Santa Cruz Biotechnology), anti–CDK4 (H-22) (catalog sc-601, Santa Cruz Biotechnology), anti–Cited1 (catalog RB-9219,
Neomarkers), anti–Claudin-2 (catalog 51-6100, Invitrogen Zymed), anti–Na+/K+-ATPase α-1 subunit (catalog 05-369, MilliporeSigma), anti-GAPDH (catalog sc-32233, Santa Cruz Biotechnology), anti-Sox9 (catalog ab185966, Abcam), anti-Pax8 (catalog ab189249, Abcam), anti-p53 (catalog sc-126, Santa Cruz Biotechnology), Phospho-p53 Antibody Sampler Kit (catalog 9919, Cell Signaling Technologies), anti-p21 (F-5) (catalog sc-6246, Santa Cruz Biotechnology), anti-p21Cip1 (catalog ab2961, Abcam), anti-TRPV5 (catalog 17322-1-AP, Proteintech), anti-ZONAB (catalog 402800, Invitrogen), anti–ZO-2 (catalog 38-9100, Invitrogen), and anti–α-acetylated tubulin (catalog T6793, MilliporeSigma). The secondary antibodies included HRP-conjugated anti-goat, anti-mouse, and anti-rabbit antibodies (catalog A0181, A0216, and A0208, Beyotime); FITC-conjugated AffiniPure donkey anti–goat IgG (H + L) (Jackson ImmunoResearch); Cy3-conjugated AffiniPure donkey anti–goat IgG (H + L) (Jackson ImmunoResearch); FITC-conjugated AffiniPure donkey anti–rabbit IgG (H + L) (Jackson ImmunoResearch); and Cy3-conjugated AffiniPure donkey anti–rabbit IgG (H + L) (Jackson ImmunoResearch).

SAβ-Gal. The SAβ-Gal staining was performed on mouse kidney sections as previously described (51, 52). SAβ-Gal provides a simple assay for chromosomes that induce senescence. Briefly, kidney sections were fixed with 3.7% paraformaldehyde in PBS and then incubated in staining solution (40 mM citric acid/phosphate, pH 5.8, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl2, 1 mg/mL X-gal) at 37°C for 16–18 hours. Next, cells were washed twice with PBS, after which they were counterstained with 1% eosin solution for 5 minutes. Finally, blue cells in kidney sections were considered positive. To detect lysosomal β-Gal, the citric acid/sodium phosphate was at pH 4.0. For each cortical region, 10 consecutive fields at original magnification ×200 per section were selected for measurement (53). Signals were quantified by digital elaboration of microscopy images using ImageJ software (NIH, https://imagej.nih.gov/ij/).

Method of cilia length measurement. Murine IMCD3 cells were maintained at 37°C in 5% CO2 in DMEM (Invitrogen) supplemented with 10% FBS. To induce ciliary formation, cells were cultured in the serum-free medium for 48 or 72 hours. The RNA oligonucleotides that specifically targeted mouse Robo2 were purchased from Santa Cruz Biotechnology. The RNA oligonucleotides were transfected with DharmaFECT siRNA transfection reagent (Dharmacon).

Cells were grown on glass coverslips and fixed with 95% cold methanol for 10 minutes at –20°C followed by permeabilization with 0.2% Triton X-100 in PBS for 20 minutes. Cells were blocked with 2% bovine serum albumin in PBS for 1 hour. Cells were incubated with anti–α-acetylated tubulin antibody (T6793, MilliporeSigma) for 1 hour at room temperature or overnight at 4°C and then with secondary antibody for 1 hour at room temperature. Cells were mounted with DAPI Fluoromount-G (Vectorlabs) and then examined with a Nikon Eclipse 80i microscope. The percentage of ciliated cells and the length of cilia were measured with the ImageJ software.

Immunofluorescent staining. The kidneys were fixed in 4% paraformaldehyde (MilliporeSigma) overnight at 4°C, and then the samples were equilibrated in 30% sucrose/PBS, 20% sucrose/PBS, and 10% sucrose/PBS at 4°C for 16 hours, 6 hours, and 4 hours, respectively. The embryos were transferred to OCT embedding medium and mounted in OCT using cryomolds. The sections were sectioned (5 μm thick) and permeabilized with 0.05% Triton X-100 and 0.1% SDS in PBS, pH 7.4. The samples were blocked with 0.1% casein (Vector Labs) and incubated overnight with the primary antibodies (diluted in 0.1% casein) at 4°C. After extensive washing, the samples were incubated with a dilution of the appropriate fluorescent dye– or HRP-conjugated secondary antibody (1:400) at 4°C for 6 hours. The sections were counterstained with DAPI. The sections were then washed and mounted for observation, and each fluorescent channel was captured separately using a scanning confocal microscope (Olympus FluoView1000).

Microarray analysis and gene expression profiling. Microarray analysis was performed by the Microarray Core Facility at the CapitalBio Corporation. Affymetrix GeneChip Mouse Genome 430 2.0 microarrays were used throughout the experiments (GEO GSE137298). The expression and functional profiles of genes were compared between the Robo2-null and wild-type kidneys. The functional profiling of the Robo2-regulated genes was performed using the Database for Annotation, Visualization, and Integrated Discovery based on the biological pathways from the Kyoto Encyclopedia of Genes and Genomes database, as previously described (54).

NanoString gene expression analysis. A total of 9 genes, including Cdkn1a, Cdkn1b, B-Raf, Cdc42, Cnma1, Ctnnb1, Pten, Tjp1, and Trp53, were selected to analyze the expression of signature genes associated with the p53 pathway and TJ pathway. NanoString quantification of these genes’ mRNA levels was performed using the nCounter analysis system and a custom-designed code set as previously described (see above).
Two specific probes (capture and reporter) for each gene of interest were used. In brief, approximately 0.1–0.2 μg of RNA was used to quantify the gene expression via the nCounter Analysis System according to the manufacturer’s instructions. The mRNA molecules were counted on a NanoString nCounter as previously described (55, 56). The data analysis was performed according to the NanoString Technologies recommendations. The mRNA counts were processed to account for hybridization efficiency, background noise, and sample content. Each sample profile was normalized to the geometric mean of 3 housekeeping genes: Actb, B2m, and Pkg1 (Figure 2B).

Mass spectrometry–based IP. The embryonic kidney proteins were isolated to identify the endogenous Robo2-interacting proteins. The protein samples were prepared as previously described (57, 58). E15 embryonic kidney proteins were isolated and extracted in ice-cold RIPA lysis and extraction buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.1% SDS; 1% NP-40; 0.5% sodium deoxycholate; 1 mM Na3VO4; 1× protease inhibitor; and 1 mM NaF). After preclearing the lysate, 2 μg of the Robo2 antibody was mixed with 1 μg of lysate and 80 μL of Protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology) and incubated overnight at 4°C to immunoprecipitate the proteins. The beads were washed 3 times with ice-cold washing buffer and resuspended in SDS loading buffer (Thermo Scientific Pierce). The samples were loaded onto an SDS-PAGE gel. The entire gel was cut for each run and divided into 4 sections according to molecular size. The cut gel was subjected to in-gel digestion, and then 2D LC/MS analysis was performed as previously described (57, 58). The data were further analyzed by the ProteinLynx Global Server version 2.5 (Waters Corporation).

RNA interference. The custom and chemically modified siRNAs were designed to target Robo2 (GenePharma), 5′-GGCCAUGAGUACUGAUGAATT-3′, 3′-UUCAUCGUACUGCCCTT-5′; Baiap2 (GenePharma), 5′-GCACUGAAGAAAUACCAAATT-3′; Tjp1 (GenePharma), 5′-GGACCAUAUGGGAATT-3′; and Ybx-3 (GenePharma), 5′-CCGAGGCCCCAACACUAUUTT-3′, 3′-AUAGUGUAGGGACGUGCTTGTT-5′. A nonspecific siRNA duplex served as a control (GenePharma). To knock down Robo2, Baiap2, Tjp1, Ybx3, ZONAB, and and Ybx-3 in vitro, the MDCK cells were transfected with the individual siRNAs using the JetPRIME Reagent (POLYPUS, 114-75).

To assess the effects of Slit2 and of silencing of Robo2 on gene expression, we starved MDCK cells overnight in DMEM/F-12 (Invitrogen) supplemented with 0.1% FBS, and cells were treated with ROBO2 siRNAs and then with 50 nM recombinant human SLIT2 N-terminus (MilliporeSigma, SRP3155) or buffer for 24 hours as previously described (59, 60). After the media were removed, cells were lysed for Western blotting.

Co-IP. To test whether Robo2 and Baiap2 interacted in vitro, we designed constructs of Robo2 and Baiap2 (a gift from Naoki Mochizuki, Department of Cell Biology, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan). Ten percent of the total lysate was reserved; the remaining was centrifuged and the supernatant was transferred to a clean microcentrifuge tube (Thermo Fisher Scientific). The lysate was precleared by incubation with Protein A/G agarose for 3 hours on a rotator at 4°C. The beads were centrifuged, and the supernatants were transferred to fresh tubes, where they were incubated with 5 μL Robo2 antibody for 1 hour before 50 μL of a homogenous Protein A/G agarose suspension was added. The samples were then incubated overnight at 4°C on a rotator. The complexes were centrifuged, the supernatant was removed, and the beads were washed twice with lysis buffer. Protein sample buffer was added and the samples were boiled for 3 minutes. The complexes were then analyzed as described in the immunoblotting section.

Pull-down assay. GST fusion proteins were purified as formerly described (61). For pull-down studies, purified full-length Baiap2-GST, Baiap2-250-GST, or Baiap2-1-250-GST fusion proteins from E. coli Rosetta (DE3) were incubated with glutathione-agarose beads in PBS buffer (pH 7.4) at 4°C with rotation and washed repeatedly with PBS buffer (pH 7.4). When appropriate, the beads were incubated with reconstituted Robo2-His or Robo2-CC3-3-His from E. coli Rosetta (DE3) overnight at 4°C, followed by a wash step in PBS buffer (pH 7.4). Bound proteins were collected by centrifugation and resolved by SDS-PAGE. Western blots used anti-GST (M20007, Abmart) and anti-HIS (M20001, Abmart).

Immunoblotting. The Western blots were performed as previously described (62, 63). The isolated kidneys were washed with ice-cold PBS, pH 7.4, and lysed with ice-cold RIPA lysis buffer at 4°C for 1 hour. The samples were centrifuged at 12,000 g for 15 minutes at 4°C. The samples were subjected to SDS-PAGE, transferred to PVDF membranes, and detected with the appropriate primary antibodies followed by HRP-conjugated antimouse, -goat, or -rabbit IgGs. The blottings signals were detected by enhanced chemiluminescence using the SuperSignal West Dura Extended Duration Substrate (Pierce).
Notably, we routinely isolated proteins from the C57BL/6 wild-type and Robo2-mutant kidneys, as well as the cultured HEK and MDCK cells. However, the amounts of the total isolated proteins were adjusted to obtain identical quantities and used as the starting materials for comparisons. The primary and secondary antibodies were used as described above. Bands were detected by enhanced chemiluminescence, and the relative protein expression level was quantified using ImageJ.

Tubular dilation/cystic index. To quantify the rate of cystic kidneys, we applied a grid of squares 13.625 μm large to sections of kidneys stained with H&E. We marked each cross with a dot, and we counted the number of dots inside the lumen of 3 litters. We determined the degree of dilatation according to criteria previously established (64, 65). Briefly, 1 dot: normal tubules; 2 dots: dilated tubules; >3 dots: cysts.

Data analysis. All experiments were performed at least 3 times (n) and the values presented as mean ± SEM. Statistical significance was determined using the 2-tailed Student’s t test. Probability (P) values are indicated with * and **, which correspond to values of 0.05 and 0.01, respectively. For all statistical comparisons, P values less than 0.05 were accepted to indicate significant differences.

Study approval. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Chinese PLA General Hospital and Medical School of Chinese PLA.

Author contributions
QL, YX, XL, and XC conceived and designed the research. QL, SC, QM, YL, HY, GG, EA, CR, KW, YZ, and XB performed experiments. GG and QL analyzed data. JY, XB, YX, XL, and XC interpreted results of experiments. QL and GC prepared figures. QL and XL drafted the manuscript. QL, YX, XL, and XC edited and revised the manuscript. QL, SC, QM, YL, HY, GG, EA, CR, KW, YZ, YB, GC, YX, XL, and XC approved the final version of the manuscript.

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