Disruption of the *Dapper3* gene aggravates ureteral obstruction-mediated renal fibrosis by amplifying Wnt/β-catenin signaling

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**Background:** Dapper3 is a negative regulator of Wnt/β-catenin signaling in cancer, but its physiological functions are largely unknown.

**Results:** Disruption of Dapper3 aggravated renal fibrosis after UUO through upregulating Dvl and Wnt/β-catenin signaling.

**Conclusion:** Dapper3 attenuates the fibrotic activity of Wnt signaling in the UUO model.

**Significance:** This study established the Dapper3 knockout mouse model and unraveled functions of Dapper3 in renal fibrosis.

**SUMMARY**

Wnt/β-catenin signaling plays key roles in embryonic development and tissue homeostasis. *Dapper3/Dact3*, one of the three members of the *Dapper* gene family, is transcriptionally repressed in colorectal cancer and may function as a negative regulator of Wnt/β-catenin signaling. To investigate its physiological functions, we generated a mouse strain harboring conditional null alleles of *Dapper3* (*Dapper3fl/fl*), and homozygous *Dapper3* deficient (*Dapper3−/−*) mice were produced after crossing with EIIα-cre transgenic mice. We found that *Dapper3* is not essential for mouse embryogenesis, postnatal survival and reproduction. However, adult *Dapper3−/−* mice exhibited a mild reduction in body weight compared with their wild-type littermates, suggesting a functional role of Dapper3 in postnatal growth. To investigate the role of Dapper3 in renal fibrosis, we employed the unilateral ureteral obstruction (UUO) model. *Dapper3* mRNA expression was upregulated in kidney after UUO. Loss of the
**Dapper3** gene enhanced myofibroblast activation and extracellular matrix overproduction in the obstructed kidney. Moreover, this aggravated fibrotic phenotype was accompanied with accumulation of Dishevelled2 and β-catenin proteins and activation of Wnt targeted fibrogenic genes. In primary renal tubular cells, Dapper3 inhibits Wnt induced epithelial-to-mesenchymal transition. Consistently, Dapper3 interacted with and downregulated Dishevelled2 protein, and attenuated the Wnt-responsive Topflash reporter expression. These findings together suggest that Dapper3 antagonizes the fibrotic actions of Wnt signaling in kidney.

Wnt/β-catenin signaling is crucial in cell fate determination during embryogenesis and tissue homeostasis maintenance after birth. The binding of Wnt ligands to their receptors Frizzled (Fzd) and low-density-lipoprotein receptor related protein 5/6 (LRP5/6) leads to recruitment and phosphorylation of Dishevelled (Dvl) (1). Dvl, the hub of Wnt signaling connecting Fzd and downstream components (2-4), is tightly regulated by Dapper1/Dact1, Inversin, NEDL1, Prickle-1, KLHL12 and pVHL-containing E3 ubiquitin ligase (5-9). Activated Dvl promotes the disassembly of the β-catenin degradation complex, resulting in the nuclear accumulation of β-catenin and transcriptional activation of Wnt target genes mediated by β-catenin/T cell factor (TCF) complex (1,10).

Dapper was first identified as a Dishevelled (Dvl/Dsh)-interacting protein by yeast two-hybrid screening in Xenopus (11). As an antagonist of both canonical and noncanonical Wnt signaling, it is required for notochord development of *Xenopus* embryos (11). The three members of the Dapper (gene symbol-*Dact*) family, i.e. Dapper1, Dapper2 and Dapper3, have been identified in zebrafish, mouse and human (5,12-15). Human Dapper1 can negatively modulate Wnt signaling by promoting Dvl degradation in the cytoplasm and disrupting the β-catenin/LEF1 complex in the nucleus (5,16). Zebrafish and mouse Dapper2 can inhibit TGF-β/Nodal signaling during mesoderm induction by promoting lysosomal degradation of type I receptors ALK4 and ALK5 (17,18). Dapper1 and Dapper2 knockout mouse models have been generated. Dapper1−/− mice died in the perinatal period with multiple physiological defects including caudal vertebrae agenesis, anorectal malformation, renal dysplasia, loss of bladder and genital tubercle (19,20). Through regulating the protein level and cellular distribution of Dvl2 (20), or controlling Vangl2 activity (19) in the primitive streak region, Dapper1 plays a critical role in planar cell polarity (PCP) signaling during mouse embryonic development. Missense heterozygote mutations of the *Dapper1* gene in fetus with neural tube defects (NTD) were reported recently, implicating mutated *Dapper1* as a risk factor for human NTD-related birth defects (21). Dapper2−/− mice displayed no apparent abnormalities but showed accelerated skin wound healing with enhanced TGF-β signaling activity, suggesting that Dapper2 functions in re-epithelialization of skin wounds by attenuating TGF-β signaling (22).

Human *Dapper3* was reported to be transcriptionally repressed through bivalent histone modifications in colorectal cancer, and its protein product Dapper3 may function as a negative regulator of Wnt/β-catenin signaling (23). Mouse *Dapper3* was broadly expressed during mouse embryogenesis and in adult tissues, especially enriched in adult brain and uterus (15). It shares 27% similarity to *Dapper1* and 24% similarity to *Dapper2* at the amino acid level. Among three members in the *Dapper* family,
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Dapper3 was the least understood and its in vivo functions have not yet been reported.

Tubulointerstitial fibrosis, characterized by excess matrix accumulation and deposition, is considered as the final outcome of progressive kidney diseases and an indicator of end-stage renal diseases (24-28). The rodent model of unilateral ureteral obstruction (UUO) has been widely employed to study the molecular basis of tubulointerstitial disease (29). Wnt signaling is activated in the process of tubulointerstitial renal fibrosis with the first evidence of Wnt4 reactivation in collecting duct epithelium and interstitial myofibroblasts in the UUO model (30,31). Wnt/β-catenin signaling is activated in tubular epithelial and interstitial cells after renal injury (32,33). All members of the Wnt ligand family except for Wnt5b, Wnt8b and Wnt9b, most of the Fzd receptor genes and all four Dickkopf (Dkk) members were found to be upregulated in the fibrotic kidney after UUO (33). Major cellular events in tubulointerstitial fibrosis include infiltration of inflammatory cells, activation of fibroblasts, epithelial-to-mesenchymal transition (EMT) and production of extracellular matrix (ECM) (34). Wnt signaling stabilizes both Snail and β-catenin proteins, both of which cooperatively control the EMT process (35). Plasminogen activator inhibitor-1 (PAI-1), a direct downstream target of Wnt/β-catenin signaling, is a critical player in the pathogenesis of chronic kidney diseases (36).

In this study, we have generated mice harboring conditional or null allele of Dapper3. The mild growth retardation in adult Dapper3−/− mice implies the role of Dapper3 in postnatal growth. Dapper3−/− kidneys after UUO showed enhanced myofibroblast activation and extracellular matrix accumulation, leading to more pronounced renal fibrosis. These phenotypes were accompanied with upregulation of Dvl2, β-catenin proteins and Wnt targeted fibrotic genes. The roles of Dapper3 in modulating Wnt signaling were also evidenced by the results that Dapper3 could interact with Dvl2, downregulate Dvl2 proteins and attenuate the expression of the Wnt-responsive Topflash reporter.

EXPERIMENTAL PROCEDURES

Generation of Dapper3+/flax and Dapper3−/− Mice — A 14 kb fragment consisting of Exon 2 through 4 of the Dapper3 gene was retrieved from the C57BL/6J-derived BAC. The 5′ loxP site was cloned upstream of Exon 2. The 3′ loxP site and an frt-neo-frt selection cassette were inserted downstream of Exon 3. The gene targeting vector was linearized with Pvu I and electroporated into B6/BLU mouse ES cells. The G418-resistant ES clones were screened by long-range PCR with Dapper3-ES-F 5′-tccaagacctgtaaagcttcca-3′ and Dapper3-ES-R 5′-aagggttattgaatatgatcgga-3′. The presence of both 5′ and 3′ loxP sites was additionally validated using PCR primers flanking the loxP site. Two positive colonies were microinjected into different C57BL/6J blastocysts to generate chimeric mice.

To inactivate the floxed Dapper3 allele, Dapper3+/flax mice were bred with Ella-cre transgenic mice (The Jackson Laboratory, USA). Depletion of Exon 2 and Exon 3 produced a stop codon TGA at the 142nd nt of Exon 4 and terminated translation in advance. Dapper3+/− mice were intercrossed to obtain homogenous Dapper3 knockout mice. This study was carried out under the approval of the Animal Research Committee of Model Animal Research Center (MARC), Nanjing University and Institutional Animal Care and Use Committee (IACUC) of Tsinghua University.

Genotyping of Dapper3 Knockout Mice — Dapper3 conditional and deleted alleles were genotyped by PCR using genomic DNA derived
from ES cells, tail tissue or embryos. PCR primers used to identify Dapper3 allele are as follows: P1F: 5'-cctgatcatcctttcattgtcccacc-3' and P1R: 5'-catctgctcctgaaaccctatt-3'; P2R: 5'-tgcaagatcccttcagagtctcc-3'.

**Unilateral Ureteral Obstruction (UUO)** — After general anesthesia, UUO was performed on both wild-type and knockout (Dapper3 +/-) male mice by ligating the left ureter using 4-0 silk after a midline abdominal incision. Sham-operated mice had their ureters exposed and manipulated, but not ligated (37). At day 7 or day 14 after surgery, the perfused kidneys were removed for analysis. One piece of a kidney was fixed in 10% formalin followed by paraffin embedding for histologic and immunohistochemical studies. The remaining was frozen in liquid nitrogen and stored at -80°C for extraction of RNA and proteins.

**Histology and Immunohistochemistry** — The 10% formalin-fixed kidneys were embedded with paraffin and sectioned at 4 µm intervals. Renal morphology was examined after they were stained with Masson's-modified trichrome stain (37). Picrosirius red staining was used for the histological assessment of collagen accumulation. Immunohistochemistry of paraffin sections was conducted with the Dako ChemMate™ EnVision System with primary antibodies for α-SMA (1:200) or β-catenin (1:200). In negative controls, primary antibodies were omitted or replaced by isotype-matched nonimmune IgG. To verify the phenotype, quantitative histology was performed and the results were confirmed by independent pathologists blinded to groups of different genotype. Images were viewed and captured using a Nikon Labophot 2 microscope equipped with a Sony CCD-IRIS/RGB color video camera attached to a computerized imaging system and analyzed by Image Pro Plus 3.0 (ECLIPSE 80i/90i; Nikon, Tokyo, Japan). The relative α-SMA positive area was calculated as a proportion of the α-SMA expressed area to total cortical area of the kidney section. For each kidney, 10 randomly selected fields were analyzed in a blinded manner.

**Southern Blot Analysis** — Genomic DNA from ES cells or mouse tissues was digested with Xba I. Approximately 10 µg digested DNA was electrophoresed on 0.7% agarose gel, transferred to a nylon membrane, and fixed by UV cross-linking. The membrane was hybridized in ExpressHyb solution (Clontech, CA, USA) for 2 hours at 65°C. The radioactively labeled blots were detected by exposure to Kodak film at -80°C for 72 hours. The 5' probe used to identify the wild-type (17.3 kb) and targeted allele (9.5 kb) from Xba I digested genome, was generated by primers 5F: 5'-ccgctttacttctgagggtctg-3' and 5R: 5'-cctgaagacacaagcagacg-3'.

**Isolation and Culture of Mouse Embryonic Fibroblasts (MEFs)** — The male and female Dapper3 +/- mice were intercrossed and the pregnant female mice were sacrificed at 13.5-days post coitum. After embryos were separated individually, the brain, limbs, tail and dark red organs of the embryos were cut away. The yolk membrane of every embryo was kept for genotyping. After washing several times with cold PBS, embryos were minced with surgical scissors in a minimal amount of trypsin and incubated at 37°C for 10 min. Then DMEM with 10% FBS was added to separate tissues until free of any larger pieces of tissue. These cells were cultured at 37°C with 5% CO₂.

**Immunoblotting Analysis** — To detect nuclear β-catenin levels, the nuclear fraction was isolated from kidney homogenate as described (16). In other cases, tissues or cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Roche Applied Science, Mannheim, Germany) for 30 min at 4°C. After centrifugation at 12,000g for 10 min, the supernatant was...
analyzed by SDS-PAGE. Immunoblotting was performed with primary antibody and secondary anti-rabbit antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Proteins were visualized by chemiluminescence. The Dapper3 polyclonal antibody was generated by immunizing rabbit with mouse Dapper3 (170-236aa) - glutathione S-transferase fusion protein. The antibodies against Collagen I, α-SMA, Dvl2 and β-catenin were purchased from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA), Sigma-Aldrich Corporation (St. Louis, MO, USA), Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Dallas, Texas, USA), respectively.

RNA Isolation and Real Time RT-PCR — Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1 μg of RNA with Revertra Ace (Toyobo, Osaka, Japan). Real time RT-PCR was performed by the EvaGreen method with the Mx3000p Quantitative PCR system (Agilent Technologies, Santa Clara, CA, USA). Gene expression was normalized against GAPDH mRNA. Each sample was measured in triplicates. The primers used were as follows:

- Col1a1: 5'-agacatgtcagttgtggac-3' and 5'-gcagctgacttcagggatg-3';
- Col3a1: 5'-aggcaaacagtggttctccg-3' and 5'-gacctcgtgctccagttagc-3';
- Dapper3: 5'-agtcgcccgccttcagct-3' and 5'-ccatcccgccccaactca-3';
- Dvl2: 5'-getccacatgccccatggc-3' and 5'-tgccagctgctggagactcaag-3';
- Axin2: 5'-gcagcagatccgggaggatgaa-3', and 5'-gattgacagccgggggtcttga-3';
- c-Myc: 5'-tgagcccctagtgctgcat-3' and 5'-agcccgcctccgacctctt-3';
- cyclin D1: 5'-egctcgtgctggagactcaag-3' and 5'-ctcctcttcgcacttctgctc-3';
- Acta2: 5'-gtccagacatccggaggtaa-3', and 5'-gattgacagccgggggtcttga-3';
- Gapdh: 5'-catggtccttcgctctcgtt-3' and 5'-gagggtctgctaacatcactg-3';
- PAI-1: 5'-cccgcctcctcatcctgcct-3' and 5'-gccactgtgccgctctcgtt-3';
- Snail: 5'-gggaagcccaacttagcgacg-3' and 5'-caggtaagatcttccgac-3';
- Slug: 5'-ccatcccgccccaactca-3';
- pE1: 5'-atgatccgggccttctcgttc-3', pE4: 5'-agtgggctaggtgtcaggaag-3';
- Slug: 5'-catccttggggcgtgtaagtc-3' and 5'-gcccagagaacgtaaataggtc-3';
- Mmp7: 5'-cgccactgtcccaggaag-3';
- PAI-1: 5'-cccgcctcctcatcctgcct-3' and 5'-gceactgtgccgctctcgtt-3';
- Snail: 5'-gggaagcccaacttagcgacg-3' and 5'-caggtaagatcttccgac-3';
- Slug: 5'-ccatcccgccccaactca-3';
- pE1: 5'-atgatccgggccttctcgttc-3', pE4: 5'-agtgggctaggtgtcaggaag-3';
- Gapdh: 5'-catggtccttcgctctcgtt-3';
- PAI-1: 5'-cccgcctcctcatcctgcct-3' and 5'-gceactgtgccgctctcgtt-3';

Immunofluorescence — Primary renal tubular epithelial cells were isolated as previously described (37). Cells were grown on glass coverslips in 6-well plate, and treated with 40ng/ml Wnt3a (R&D Systems, Minneapolis, MN) for 72 hours. Subsequently, the cells were fixed and stained for E-cadherin (Abcam, Cambridge, UK), α-SMA and fibronectin (Santa Cruz Biotechnology, Dallas, USA). For co-localization assay, HeLa cells were transfected with Flag-Dvl2 together with Myc-Dapper3 for 36 hours, and detected by indirect anti-Flag or anti-Myc immuno-fluorescence. Nuclei were counter-stained with DAPI. Images were taken with Leica TCS SP5 Confocal Laser Scanning Microscope.

Luciferase Reporter Assays — HEK293T cells were transfected with various plasmids as indicated. At 36 hours post-transfection, the cells were harvested, and luciferase activities were measured by aluminometer (Berthold Technologies, Bad Wildbad, Germany). Reporter activity was normalized to the co-transfected Renilla. Experiments were repeated in triplicate, and the data were represented in the mean ± SD.

Statistical Analysis — For statistical analysis, χ² test was used to determine whether genotype distribution of intercrossed offspring was in line with the expected Mendelian ratio. Independent Student's t test (two tailed) was performed for comparison of body weight, histology, protein, and mRNA data between wild-type and Dapper3 deficient mice. The P value less than 0.05 was regarded as statistically significant and indicated...
RESULTS

Generation of Dapper3 Conditional Knockout and Null Allele Mice — To investigate the physiological functions of Dapper3, we generated conditional Dapper3 knockout mice with the Cre/loxP homologous recombination system. The Exon 2 and 3 of the Dapper3 gene were flanked with the loxP sites for Cre-mediated deletion to produce a stop codon at the 142nd nt of Exon 4 (Fig. 1A). The long-range PCR identified six B6/BLU ES cells colonies with a successful recombination event. They were further verified by Southern blotting with XbaI (Fig. 1B) or BamH I digested genomic DNA (data not shown). The presence of both the 5’ and 3’ loxP sites were confirmed using PCR primers flanking them. The intercrossed offspring of Dapper3+/loxP F1 mice could be distinguished by PCR genotyping (Fig. 1C). Dapper3+/loxP mice and EIIa-cre transgenic mice were mated to produce Dapper3+/- embryos (Fig. 1D). The heterozygous Dapper3+/- mice were intercrossed to produce Dapper3-/- embryos (Fig. 1E). To confirm the genotypes, we examined the Dapper3 mRNA and protein expression in mouse embryonic fibroblasts (MEFs). As expected, RT-PCR with primers in Exon 1 and Exon 4 revealed a 656-bp fragment in Dapper3+/- MEFs and a 406-bp truncated fragment in Dapper3-/- MEFs (Fig. 1E). Western blot analysis confirmed the absence of a full-length Dapper3 protein in MEFs (Fig. 1F). These data together demonstrate that we have successfully generated Dapper3 conditional knockout and null allele mice.

Adult Dapper3-/- Mice Showed Mild Growth Retardation. — Dapper3-/- mice are viable and fertile. Of 156 offspring from Dapper3+/- intercross, 45 (29.0%) were wild-type, 81 (52.1%) were heterozygous, and 30 (18.9%) were homozygous for the Dapper3 allele, accordant with the Mendelian ratio by \( \chi^2 \) analysis (\( P=0.178 \)). To investigate whether Dapper3-/- knockout has influence on mouse postnatal growth, Dapper3+/+ and wild-type littermates were weighed at an average interval of 2 weeks for 6 months and their growth curves were drawn (Fig. 2A). Female Dapper3-/- mice exhibited a small reduction (6.5% on average) in body weight compared with their wild-type littermates. The significant difference was observed in some of early points of growth, reaching the largest at about 17 weeks old. However, after that, no significant different can be seen between Dapper3-/- and wild-type females. In contrast, Dapper3-/- males showed a significant lag in weight gain comparing with wild-type mice from 17 weeks old on, and this difference increased gradually with age from 5.0% reduction to 8.6%. It is unlikely due to the skeleton growth defects as there is no significant difference in body length between wild-type and knockout mice (data not shown). The mild growth retardation in Dapper3-/- mice, especially in males, implies that Dapper3 may play a role in normal growth of adult mice.

The difference in body weight prompted us to examine whether it is due to defects of glucose and lipid metabolism. Glucose tolerance test (GTT) showed that both wild-type and knockout mice could quickly clear the redundant glucose from blood (Fig. 2B). Although the glucose values in Dapper3-/- mice are lower at each time points, there is no statistical significance. Measurement of several parameters in mouse serum revealed that the Dapper3-/- mice have no serious problem in lipid metabolism as well as functions of liver and kidney (Table 1).

Renal Fibrosis is Aggravated in Dapper3-/- Mice after UUO. — To investigate the role of Dapper3 in physiological/pathological condition, we employed UUO-induced kidney fibrosis model as we and others have reported that the Wnt/Snail
pathway are involved in kidney fibrosis (32,33,37). We found Dapper3 mRNA was upregulated after UUO (Fig. 3A). Importantly, interstitial fibrotic area was significantly increased in Dapper3−/− kidneys 7 days after UUO compared with that in wild-type ones, as shown by Masson's trichrome staining (Fig. 3B and 3C). UUO led to a marked increase in collagen accumulation and deposition in wild-type mice, which was detected by a collagen-specific Picrosirius red staining, and disruption of the Dapper3 gene further enhanced the collagen deposition visualized by greater red staining area (Fig. 3D). Consistently, the expressions of Col1a1 and Col3a1, encoding type I and type III collagen respectively, were significantly increased in Dapper3−/− obstructed kidneys (Fig. 3E and 3F). The collagen I protein level was significantly higher in the Dapper3−/− obstructed kidneys than that in wild-type kidneys at Day 14 of UUO (Fig. 3G). These results strongly suggest that disruption of the Dapper3 gene accelerates myofibroblast activation, EMT and matrix production in obstructed kidney, leading to the enhanced fibrotic phenotypes in the UUO model.

**Promotion of Dvl and β-catenin Accumulation as well as Wnt Target Gene Expression in Dapper3−/− Obstructed Kidneys** — Wnt signaling plays a critical role in kidney fibrosis (32,33). To explore the molecular mechanism underlying the fibrosis phenotype in the Dapper3−/− obstructed kidneys, we examined the influence of Dapper3 on renal β-catenin abundance after obstructive injury. As shown in Figure 5A, UUO caused a marked induction and time-dependent increase of β-catenin protein levels predominantly in renal tubules, and a stronger β-catenin induction was observed in Dapper3−/− kidneys than Dapper3+/+ controls at both 7 days and 14 days after UUO. In addition to being found at the cell-cell adhesions, β-catenin was also localized in the cytoplasm and the nuclei of tubular epithelial cells and much more nuclear β-catenin was observed in Dapper3−/− samples (Fig 5A, arrowheads), indicating enhanced Wnt signaling. Western blot also confirmed that both the total β-catenin protein level (Fig. 5B) and nuclear β-catenin (Fig. 5C) was significantly upregulated in Dapper3−/− obstructed kidneys. Dvl2, another core Wnt pathway component upstream of β-catenin, was not found to be significantly increased in Dapper3−/− kidneys without injury, perhaps due to the low expression of endogenous protein. However, the increased Dvl2 protein levels were noticed in Dapper3−/− kidney than in wild-type ones after UUO (Fig. 5B).
As the Dvl2 mRNA level was unchanged (data not shown), these data suggest that Dapper3 may regulate Dvl2 at the protein level in renal fibrosis. Supporting this note, classical Wnt target gene Axin2, c-Myc and cyclin D1 were all upregulated in Dapper3−/− fibrotic kidney (Fig. 5D-F). All these results indicate that loss of Dapper3 aggravates ureteral obstruction-induced renal fibrosis through upregulating Wnt/β-catenin signaling.

**Dapper3 inhibits Wnt-induced EMT in renal tubular cells.** — EMT of tubular epithelium could contribute to the pool of matrix-producing cells in renal fibrosis, in which Wnt signaling serves as an important promoter. We then examined the role of Dapper3 in EMT in primary cultured renal tubular cells. Treatment of cells with Wnt3a significantly decreased the level of the epithelial marker E-cadherin, and increased the level of mesenchymal cell markers, fibronectin and α-SMA (Fig. 6A). Moreover, in cells from Dapper3 KO mice, Wnt-induced changes of EMT markers were more prominent compared with that in wild-type cells, suggesting that loss of Dapper3 enhanced Wnt-induced EMT in renal tubular cells.

**Dapper3 Downregulates Dvl2 Protein Levels and Wnt Signaling Activity.** — The above observations suggest that Dapper3 is a negative regulator of Wnt/β-catenin signaling. To further investigate it, we examined the effect of Dapper3 on Wnt/β-catenin signaling in HEK293T cells. As shown in Fig. 6B and 6C, Dapper3 interfered with the Wnt3a- and Dvl2-induced expression of the Wnt-responsive Topflash- luciferase reporter in a dose-dependent manner. As Dapper3 inhibited Wnt signaling activity at the level of Dvl, we next studied the relationship of these two proteins. Indeed, Flag-Dvl2 was found to interact with HA-Dapper3 (Fig. 6D), which was further consolidated by immunofluorescence analysis showing that Dapper3 was colocalized with Dvl2 in the cytoplasm (Fig. 6E). Our previous work demonstrated that Dapper1 could promote Dvl degradation(5). To test whether Dapper3 has the similar effect, HEK293T cells were co-transfected with Flag-Dvl2, Myc-Dapper3 along with GFP as a control. As shown in Fig. 6F, Dapper3 expression reduced Dvl2 protein levels in a dose-dependent manner. These results together indicate that Dapper3 negatively regulates Wnt/β-catenin signaling through interacting and downregulating Dvl2 in the cytoplasm.

**DISCUSSION**

Our results indicate an important role of Dapper3 in UUO-induced renal fibrosis by regulating the Wnt/b-catenin signaling pathway (Fig. 7). Wnt/β-catenin signaling is activated by UUO and contributes to renal fibrosis. Dapper3 negatively regulates Wnt signaling by inducing Dvl degradation. Dapper3 knockout leads to the upregulation of Dvl and thus enhanced Wnt/β-catenin signaling, leading to further activation of fibrotic related target genes and aggravated renal fibrosis.

All the three members of the Dapper family have been targeted for gene deletion, but the phenotypes of the knockout mice are very distinct. Dapper1 knockout mice are perinatal lethal with multiple urogenital defects, whereas Dapper3 was not essential for mouse embryogenesis. The mild growth retardation in adult Dapper3−/− mice implies a possible function in mouse postnatal growth, but there were no obvious defects either in skeleton growth defect, glucose and lipid metabolism, or in motor ability on the treadmill (data not shown). Therefore, the exact role of Dapper3 in this process requires further investigation. During the course of this study, we observed a young Dapper3−/− mouse suffering from polycystic kidney disease and renal fibrosis, while all the other Dapper3−/− mice did not exhibit renal dysfunction or pathological changes spontaneously,
However, a 2.4-fold induction of Dapper3 mRNA expression level was observed in kidneys after UUO, implying a potential role of Dapper3 in UUO-induced renal fibrosis. Consistent with this hypothesis, Dapper3 knockout mice exhibited more severe kidney fibrosis after UUO, as shown by the increase in collagen accumulation and induction of α-SMA and other fibrotic genes.

Wnt signaling plays a critical role in organogenesis and tissue homeostasis of kidney, and its over-activation can cause fibrosis and progressive renal failure (40). In Dapper3⁻/⁻ kidney, Wnt signaling was more activated than wild-type controls upon UUO. Our data provided multiple supports for this conclusion, including increased protein levels of Dvl and β-catenin, enhanced nuclear localization of β-catenin, and stronger induction of the Wnt targets Axin2, c-Myc and cyclin D1 in Dapper3⁻/⁻ mice. Among these Wnt targets, c-Myc was most enhanced by UUO injury (33), and was further stimulated by loss of Dapper3.

Wnt signaling is also a key mediator during the fibrosis-related EMT process. The EMT marker genes PAI1, Snail, Slug were all significantly increased in Dapper3⁻/⁻ kidneys compared to wild type ones after UUO. In primary cultured renal tubular cells, Wnt3a treatment decreased the level of the epithelial marker E-cadherin and increased the mesenchymal markers fibronectin and α-SMA, and Dapper3 ablation further amplified this phenomena. These data indicate that loss of Dapper3 promotes renal fibrosis through deregulating Wnt-regulated EMT event. In Dapper3⁻/⁻ obstructed kidneys, we found the expression levels of the interstitial matrix related genes Fibronectin, Mmp7 and PAI1 were all raised. Fibronectin expression has been shown to be controlled by β-catenin (41), MMP-7 is regulated by Wnt4 and Wnt1 in the kidney (42), and PAI-1 was identified as a direct downstream target of Wnt/β-catenin signaling moderating the fibrotic action of Wnt signaling (36). Therefore, it is conceivable that Dapper3 may antagonize the fibrotic actions of Wnt signaling through repressing its target genes.

Mechanistically, Dapper3 may function as a negative regulator of Wnt/β-catenin signaling as Dapper1 does. Dapper3 could reduce Topflash-luciferase expression induced by both Wnt3a and Dvl2 in a dose-dependent manner. Consistently, Dapper3 promoted Dvl2 degradation. Our findings were in agreement with the inhibitory effect of Dapper3 on Wnt signaling in colorectal cancer (23).

Targeting Wnt/β-catenin signaling might be an effective strategy to hinder the progression of renal interstitial fibrosis (32,33). The identification of the novel antifibrotic factor Dapper3 may yield new opportunity for therapeutic interventions of renal fibrosis.

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FOOTNOTES
The abbreviations used are: Dact, Dishevelled-associated antagonist of β-catenin; UUO, unilateral ureteral obstruction; Dvl, Dishevelled; EMT, epithelial-to-mesenchymal transition; PAI-1, Plasminogen activator inhibitor-1; Mmp7, Matrix metallopeptidase 7; α-SMA, α-smooth muscle actin; Fn, fibronectin; WT, wild type; KO, knockout

FIGURE LEGENDS
FIGURE 1. Generation of mice harboring Dapper3neo-flox or Dapper3 null allele. (A), Schematic diagram of the wild-type Dapper3 allele, targeting vector, targeted Dapper3neo-flox, and Dapper3 null allele. Boxes with numbers represent exons. Arrows mark the position of the PCR primers used for genotyping. The location of the 5' outside and 3' outside probes for Southern blot analysis are indicated. Solid triangles denote loxP sites; open triangles denote frt sites. Neo and TK are positive and negative selection genes, respectively. Besides restriction site Pvu I, two additional sites for Southern blot were introduced...
in the targeting vector: a BamH I site between 5' loxP site and Exon 2 and an Xba I site between 3' loxP site and Exon 4. The 5' homologous arm of the targeting vector is 5.7 kb, and the 3' arm is 4.5 kb. Dapper3\textsubscript{neo-flox} allele is created through homologous recombination between wild-type allele and targeting vector. Dapper3 null allele was conversed from Dapper3\textsubscript{neo-flox} allele by crossing to transgenic cre mice. (B), Southern blot analysis of Xba I digested genome DNA from positive ES cell clones. A 5' outside probe was used to identify the wild-type (17.3 kb) and targeted allele (9.5 kb) from Xba I digested genome DNA. The Dapper3\textsuperscript{+/fl} ES cell clones show both 17.3-kb and 9.5-kb bands as expected. wt, wild type; fl, flox. (C), PCR genotyping of mice with Dapper3\textsubscript{neo-flox} allele. The primer set P1F-P1R was used to distinguish the targeted allele (594 bp) from the wild-type allele (497 bp). +/+, wild-type; +/-fl, Dapper3\textsuperscript{+/fl}; fl/fl, Dapper3\textsuperscript{fl/fl}. (D), Generation of mouse embryos harboring Dapper3 null allele. The combination of primer sets P1F-P1R and P1F-P2R could identify three genotypes: Only one 497-bp band represents Dapper3\textsuperscript{+/+} embryo; only one 434-bp band indicates Dapper3\textsuperscript{−/−} embryo; embryos with both the 497-bp and the 434-bp bands are Dapper3\textsuperscript{+/−}. (E), Genotyping of MEFs cells by RT-PCR. The primer set pE1-pE4 was used to amplify the wild-type Dapper3 (656 bp) or the truncated Dapper3 (406 bp). Gapdh (100bp) is used as an internal control. (F), Immunoblotting of mouse Dapper3 protein from Dapper3\textsuperscript{+/+}, Dapper3\textsuperscript{+/−} and Dapper3\textsuperscript{−/−} MEFs cells. The arrow indicates the position of Dapper3. The band below is a none-specific protein.

**FIGURE 2. Mild growth retardation in adult Dapper3\textsuperscript{−/−} mice.** (A), Growth curves of female (n=7) or male (n=9) Dapper3\textsuperscript{+/+} and Dapper3\textsuperscript{−/−} mice. *, \(P<0.05\); **, \(P<0.01\). The Dapper3\textsuperscript{+/+} and Dapper3\textsuperscript{−/−} mice from 5 weeks to 30 weeks old were weighed at an average interval of 2 weeks. (B), Intraperitoneal glucose tolerance test (GTT) during 120 min in both female and male mice. The tests were conducted on 8-month-old female and 6-month-old male mice (n=7).

**FIGURE 3. Renal fibrosis is aggravated in Dapper3\textsuperscript{−/−} mice after UUO.** (A), Dapper3 mRNA levels from the kidneys of Sham-operation and UUO mice for 14 days were detected by real-time RT-PCR. (B), Masson's trichrome staining of wild-type and Dapper3\textsuperscript{−/−} kidney sections from Sham-operated, 7-day and 14-day UUO groups. Scale bars: 100 µm. (C), Relative fibrotic area statistics of Masson's trichrome staining in (B). *, \(P<0.05\). n=3. (D), Picrosirius red staining of kidney sections of wild-type and Dapper3\textsuperscript{−/−} mice after UUO for 7 days. Scale bars: 100 µm. (E, F), Relative mRNA levels of Col1a1 (E) and Col3a1 (F) from wild-type and Dapper3\textsuperscript{−/−} kidney after Sham-operation or UUO for 14 days. (G), Western blot of collagen I protein in contralateral and 14-day UUO samples. GAPDH is used as a loading control. Quantification of collagen I protein levels relative to GAPDH is shown below each band.

**FIGURE 4. Dapper3 deficiency enhances myofibroblast activation and EMT in kidneys after UUO.** (A), Representative diagrams of α-SMA immunostaining in WT and Dapper3\textsuperscript{−/−} kidneys 7 days after Sham-operation or UUO. Scale bars: 100 µm. (B), Relative α-SMA-positive area was calculated from immunostaining pictures in (A) (see the Experimental Procedures for the details). *, \(P<0.05\). n=3. (C, D), Western blots (C) and quantification analysis (D) of α-SMA protein levels in contralateral and 7-day UUO samples. GAPDH is used as an internal control. **, \(P<0.01\). n=4. (E), Effects of Dapper3 knockout
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on various fibrotic gene expression. Wild-type and Dapper3−/− mice were subjected to UUO, and after 14 days the relative transcript levels of genes involved in renal fibrosis were measured.

**FIGURE 5. Enhanced activation of the Wnt/β-catenin canonical pathway in Dapper3−/− obstructive kidneys.** (A), Immunohistochemical staining of β-catenin in wild-type and Dapper3−/− kidneys in different groups: Sham controls, 7 days after UUO and 14 days after UUO. Two representative areas with β-catenin staining in 14-day UUO group are enlarged in the right boxes. Nuclear localization of β-catenin in tubular epithelial cells (arrowheads) is increased in Dapper3−/− kidneys after UUO than that in wild-type ones. Scale bars: 100 µm. n=3. (B), Western blot analysis shows further increase in renal β-catenin and Dvl2 levels 7 days after obstructive injury in Dapper3−/− group. Quantification of β-catenin and Dvl2 protein levels relative to GAPDH is shown below each band. n=4. (C), Western blot of β-catenin in the nuclear extracts from kidney tissues shows increased nuclear β-catenin levels in Dapper3−/− group at Day 14 after obstructive injury. Quantification of β-catenin protein levels relative to the nucleus marker lamin B is shown below each band. Tubulin is used as the cytosolic marker. (D-F), Relative mRNA levels of Axin2 (D), c-Myc (E) and cyclin D1 (F) genes in wild-type and Dapper3−/− mice 14 days after Sham-operation or UUO. **, P<0.01.

**FIGURE 6. Dapper3 inhibits Wnt signaling.** (A), Immunofluorescence staining of E-cadherin, α-SMA and fibronectin in renal tubular cells. Primary renal tubular epithelial cells from Dapper3 wild-type and KO mice were treated with 40ng/ml Wnt3a for 72 hours and stained for E-cadherin (red), α-SMA (green) and fibronectin (green). Nuclei were counter-stained with DAPI (blue). Scale bars: 50 µm. (B, C), Dapper3 inhibits Wnt3a- (B) or Dvl2-(C) activated expression of TopFlash-luciferase reporter in a dose-dependent manner. HEK293T cells were co-transfected with reporter plasmid (0.1 µg), the constructs encoded Wnt3a (0.1 µg) or Dvl2 (0.1 µg) with or without Dapper3 (50 ng-200 ng). At 36 h post-transfection, the cells were harvested for luciferase assay. Experiments were repeated in triplicate. (D), Dapper3 interacts with Dvl2. HEK293T cells were transfected with HA-Dapper3 and Flag-Dvl2 as indicated. At 36 h post-transfection, the cells were harvested for anti-HA immunoprecipitation and anti-Flag immunoblotting (upper panel, arrow). Protein expression was confirmed by immunoblotting with the total cell lysates (middle and lower panels). Asterisk indicates IgG heavy chain. (E), Subcellular co-localization of exogenous Dapper3 and Dvl2. HeLa cells were transfected with Flag-Dvl2 together with Myc-Dapper3. Subcellular localization of Dvl2 (red) or Myc-Dapper3 (green) was detected by indirect anti-Flag or anti-Myc immunofluorescence. Their co-localization was shown in the merged images (yellow), and the nuclei were counter-stained with DAPI (blue). Scale bars: 10 µm. (F), Dapper3 could effectively reduce the protein level of Dvl2. HEK293T cells were co-transfected with Flag-Dvl2 (0.2 µg), gradient Myc-Dapper3 (0.3-1.0 µg) along with GFP (0.2 µg) as a control. At 36 h post-transfection, the cells were harvested for immunoblotting (IB).

**FIGURE 7. Schematic diagram illustrates the role of Dapper3 in UUO-induced renal fibrosis by regulating Wnt/β-catenin signaling.** The Wnt/β-catenin pathway is activated by UUO and contributes to renal fibrosis. Dapper3 induces Dvl degradation and thus attenuates Wnt signaling. Ablation of Dapper3 leads to increased protein levels of Dvl and Wnt/β-catenin signaling, further activating fibrotic gene
expression and causing aggravated renal fibrosis.
Table 1. Serum biochemistry parameters of male \textit{Dapper3}\textsuperscript{+/+} and \textit{Dapper3}\textsuperscript{-/-} mice.\textsuperscript{a}

| Parameter | BUN (mmol/L) | CREA (mg/dL) | UA (µmol/L) | ALB (g/L) | ALP (IU/L) | AST (IU/L) | ALT (IU/L) | CHOL (mmol/L) | TG-B (mmol/L) |
|-----------|--------------|--------------|-------------|-----------|------------|------------|------------|--------------|---------------|
| \textit{Dapper3}\textsuperscript{+/+} | 9.844 ± 1.966 | 0.411 ± 0.060 | 156.333 ± 8.327 | 16.333 ± 1.658 | 61.222 ± 11.883 | 48.714 ± 14.233 | 47.286 ± 11.354 | 2.132 ± 0.340 | 0.807 ± 0.218 |
| \textit{Dapper3}\textsuperscript{-/-} | 9.640 ± 1.405 | 0.440 ± 0.070 | 140.333 ± 6.506 | 16.556 ± 1.236 | 64.444 ± 10.899 | 49.429 ± 4.826 | 48.889 ± 17.794 | 2.214 ± 0.538 | 0.774 ± 0.313 |
| \textit{P value} | 0.798 | 0.350 | 0.138 | 0.751 | 0.557 | 0.902 | 0.839 | 0.701 | 0.797 |

\textsuperscript{a} These mice are 7-month old. Data are mean ± SD (n=9). There was no statistically significant difference in all parameters between WT and KO groups.
Figure 1

A

WT allele

Targeting vector

Dapper3\textsuperscript{neo-fox}

Dapper3 ko

B

Xbal Digest

ES: +/- +/flopx

wt

tox

17.3

9.5

C

Mouse: +/- +/- +/- +/- flox flor bp

D

Mouse: +/- +/- +/- +/- bp

E

MEF: +/- +/- +/- +/- bp

wt Dapper3

trun Dapper3

F

MEF: +/- +/- +/- +/- bp

IB:

a-Dapper3

Gapped
Dapper3 moderates renal fibrosis in mice

Figure 2

A

Female

Body weight (g)

Male

Body weight (g)

5 10 15 20 25 30

5 10 15 20 25 30

Age (weeks)

Age (weeks)

Dapper3+/+

Dapper3-/-

Dapper3+/+

Dapper3-/-

B

Female

Blood glucose (mmol/L)

Male

Blood glucose (mmol/L)

0 20 40 60 80 100 120

0 20 40 60 80 100 120

Time (min)

Time (min)
**Figure 3**

**A**  
Dapper3 moderate renal fibrosis in mice

**B**  
Sham UUO D7 UUO D14

**C**  
Masson

**D**  
Sham UUO

**E**  
Col 1a1

**F**  
Col 3a1

**G**  
Dapper3

Sham UUO

Collagen I

GAPDH

Col I/GAPDH

1.0 1.0 0.9 1.1 2.7 4.0 1.7 1.1
Figure 4

A. Relative α-SMA Positive Area

B. Sham UUO

Dapper3+/+ Dapper3-/-

C. Dapper3+/+ Dapper3-/-

Sham UUO

α-SMA GAPDH

α-SMA/GAPDH

1.1 1.0 1.2 0.9 1.6 1.3 1.4 1.4

D. Relative Protein Level

α-SMA

UO WT KO

E. Normalized mRNA level

UUO Acta2 Fn1 Vim Mmp7 PAI1 Slug Snail

** ** ** ** **
Figure 5

A

Sham

UOO D7

UOO D14

Dapper3"++"

Dapper3"-/-"

B

| Dapper3 | Sham | UOO |
|---------|------|-----|
| +/+     | -/-  |     |
| β-catenin |     |     |
| β-cat/GAPDH | 0.8 | 0.4 |
| Dvl2     |     |     |
| Dvl2/GAPDH | 0.8 | 0.4 |
| GAPDH    |     |     |

C

| Dapper3 | UOO |
|---------|-----|
| +/+     |     |
| -/-     |     |
| β-catenin |     |
| Lamin B | 1   |
| Tubulin | 3.3 |
| β-cat/Lamin B |     |

D

| Axin2 | Normalized mRNA level |
|-------|-----------------------|
| Sham  | Dapper3"++" | Dapper3"-/-" |
| UOO   | * | NS |

E

| c-Myc | Normalized mRNA level |
|-------|-----------------------|
| Sham  | Dapper3"++" | Dapper3"-/-" |
| UOO   | * | ** |

F

| cyclinD1 | Normalized mRNA level |
|----------|-----------------------|
| Sham     | Dapper3"++" | Dapper3"-/-" |
| UOO      | *** | *** |
Figure 6

A

WT | KO | WT+Wnt3a | KO+Wnt3a
---|---|---|---
E-cadherin | [image] | [image] | [image] | [image]
Fibronectin | [image] | [image] | [image] | [image]
α-SMA | [image] | [image] | [image] | [image]

B

| Condition | Relative Luciferase Activity |
|-----------|----------------------------|
| Wnt3a     |                           |
| Dapper3   |                           |
| Top-Luc, 293T | 160 ± 5 120 ± 10 60 ± 5 + |

C

| Condition | Relative Luciferase Activity |
|-----------|----------------------------|
| Dvl2      |                           |
| Dapper3   |                           |
| Top-Luc, 293T | 12 ± 0.5 6 ± 0.3 3 ± 0.2 + |

D

| Condition | Western Blot |
|-----------|--------------|
| HA-Dapper3 | [image] |
| Flag-Dvl2  | [image] |
| IP: α-HA   | [image] |
| IB: α-Flag | [image] |
| IB: α-HA   | [image] |

E

[images of DAPI, Myc-Dapper3, Flag-Dvl2, Merged]

F

| Condition | Western Blot |
|-----------|--------------|
| Flag-Dvl2  | [image] |
| Myc-Dapper3 | [image] |
| GFP        | [image] |

α-Flag | [image]
α-Myc  | [image]
α-GFP  | [image]
α-tubulin | [image]
Figure 7

Dapper3 moderates renal fibrosis in mice

Figure 7

Dapper3 moderates renal fibrosis in mice

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Dapper3 moderates renal fibrosis in mice

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Dapper3 moderates renal fibrosis in mice
Disruption of the Dapper3 gene aggravates ureteral obstruction-mediated renal fibrosis by amplifying Wnt/β-catenin signaling
Hua Xue, Zhicheng Xiao, Jing Zhang, Jun Wen, Yuan Wang, Zai Chang, Jing Zhao, Xiang Gao, Jie Du and Ye-Guang Chen

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