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Research Paper

Disruption of *Gen1* Causes Congenital Anomalies of the Kidney and Urinary Tract in Mice

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Abstract

Congenital anomalies of the kidney and urinary tract (CAKUT) are among the most common developmental defects in humans. Despite of several known CAKUT-related loci (*HNF1B*, *PAX2*, *EYA1*, etc.), the genetic etiology of CAKUT remains to be elucidated for most patients. In this study, we report that disruption of the Holliday Junction resolvase gene *Gen1* leads to renal agenesis, duplex kidney, hydronephrosis, and vesicoureteral reflux (VUR) in mice. GEN1 interacts with SIX1 and enhances the transcriptional activity of SIX1/EYA1, a key regulatory complex of the GDNF morphogen. *Gen1* mutation impairs *Grem1* and *Gdnf* expression, resulting in excessive ureteric bud formation and defective ureteric bud branching during early kidney development. These results revealed an unidentified role of GEN1 in kidney development and suggested its contribution to CAKUT.

Introduction

Congenital anomalies of the kidney and urinary tract (CAKUT) occur in three to six per 1000 live births and serve as the leading cause of renal failure in infants and children (1-3). CAKUT cover a wide range of structural malformations of the kidney and/or the urinary tract, such as renal agenesis, duplex kidneys or ureters, vesicoureteric reflux, pelviureteric junction obstruction, and ureterovesical junction obstruction. Monogenic causes of CAKUT are supported by familial clustering as well as mouse genetic models (4). To date, more than twenty disease-causing genes have been identified for human CAKUT and mutations in 12 dominant CAKUT genes are reported to be responsible for over 6% of families with isolated CAKUT (5). However, most CAKUT patients still lack any molecular diagnosis (5-7).

CAKUT result from abnormal nephrogenesis. In mammals, kidney and urinary tract development is initiated upon the specification of metanephric mesenchyme (MM) in the nephrogenic cord (NC), followed by reciprocal interactions between MM and the adjacent nephric duct (ND) (8). A single ureteric bud (UB) is firstly induced on ND by signals from MM, then invades into and branches in MM to form the future collecting duct system. At the same time, MM cells are specialized by signals from UB tips to form future nephrons and fuse with the collecting duct (9). The formation and initial branching of UBs

are critical for the development of the kidney and urinary tract (9). Blockage of either process could result in solitary kidney or renal agenesis, while extra UB formation usually leads to duplex kidney or ureters (3, 9).

During the early stages of kidney development, the secreted growth factor GDNF is expressed by MM to interact with its cognate receptor RET/GFRα-1 on ND to induce UB formation and branching (10, 11). GDNF expression is positively regulated by a group of transcription factors such as SIX1, SIX2, SIX4, PAX2, and EYA1. Disruption of any of these genes would block kidney development in mice (12-15). Meanwhile, GDNF expression are restricted by SLIT2/ROBO2 pathway that regulates the separation between ND and NC, such that supernumerary kidneys were observed in *Robo2* mutants (16, 17). GDNF signal is transduced by the negative regulator Spry1, which acts downstream of Ret (18, 19). In addition to GDNF, Spry1 also modulates FGF signals. Deletion of *Fgf10*, which is expressed during the period of UB formation, caused less severe renal agenesis than *Gdnf* or *Ret* mutations. As expected, the renal phenotype in *Fgf10* mutants could be rescued by heterozygous *Spry1* deletions (19). BMPs provide another type of signals to UB development. In particular, BMP4 is expressed in stromal cells enveloping ND to inhibit UB branching but promote ureteric stalk elongation (20). BMP4 activity is locally restricted by GREMLIN1 (GREM1), a DAN domain protein that preferentially binds to BMP2/4 (21). GREM1-mediated reduction of BMP4 activity is essential to initiate UB outgrowth (22, 23). Loss of *Grem1* causes arrested kidney development prior to UB invasion into MM (22).

The Rad2/XPG family member GEN1 was initially identified as a Holliday junction resolvase in mammalian cells (24). It has been shown that GEN1 is involved not only in DNA homologous recombination, but also in maintaining centrosome integrity (24-26). To explore the physiological role of *Gen1*, we analyzed mice carrying an insertion mutation in this gene. Here we report that disruption of *Gen1* affects the expression of both *Gdnf* and *Grem1* in mice, and caused multiple congenital anomalies of the kidney and urinary tract. These results revealed an unidentified role of GEN1 in kidney development and suggested its contribution to CAKUT.

Results

Gen1 mutants display multiple congenital defects in the kidney and urinary tract

We identified a *Gen1* mutation (*Gen1*^{PB}) in a large-scale mutagenesis project in mice (27, 28). This allele carries a *piggyBac* (*PB*) transposon insertion in the second intron (Fig. 1A). Real-time RT-PCR detected severely compromised gene expression in the mutant mice. *Gen1* transcription in homozygous (*Gen1*$^{PB/PB}$) and heterozygous (*Gen1*$^{PB/+}$) embryos was reduced to 11.6% and 62.6% of that in the wild type, respectively (Fig. 1B).

Gen1$^{PB/PB}$ mice are viable and fertile, but frequently developed kidney and urinary tract defects (Fig. 1C-O). We detected thirty-one abnormal individuals from sixty newborn pups by ultrasonic imaging. Varied and sometimes overlapped morphological defects were further revealed by following anatomical and histological analysis (Fig. 1C). We found unilateral duplex kidneys in fifteen mutants (Fig. 1F, J, N), bilateral duplex kidneys in one mutant, unilateral renal agenesis in eleven mutants (Fig. 1E, I, M), and unilateral hydronephrosis in eight mutants (Fig. 1G, K, O). The duplex kidneys in *Gen1*$^{PB/PB}$ mice are bi-papillary (Fig. 1J). Tubular elements and glomeruli were readily detected in solitary or duplex kidneys, but not in severe hydronephrotic kidneys. In addition to these morphological changes, we also observed two cases of unilateral vesicoureteric reflux (VUR) in nine *Gen1*$^{PB/PB}$ mice at weaning stages (Supplementary Fig. 1A and B). *Gen1*$^{PB/+}$ mice have similar but weaker malformations (Fig. 1C). We only detected eleven unilateral duplex kidneys from forty-nine newborn *Gen1*$^{PB/+}$ pups. No renal agenesis or hydronephrosis were observed. We also checked the renal function by measuring blood urea nitrogen (BUN) and creatinine concentration in the serum of four-month *Gen1*$^{PB/PB}$ mice. No significant differences were observed for both parameters (Supplementary Fig. 1C and D).

Other than the kidney and urinary tract defects, *Gen1*$^{PB/PB}$ mice have kinky tails as a result of delayed neural tube closure (Supplementary Fig. 2). All congenital defects were caused by the *PB* insertion. Generating revertant animals by removing *PB* in *Gen1* could result in normal animals with no kidney and urinary tract defects or delayed neural tube closure (Supplementary Fig. 3).

Excessive UB formation accompanied by altered *Grem1* and *Gdnf* expression in *Gen1* mutants

The congenital kidney and urinary tract malformations observed in *Gen1*^{PB} mutants were reminiscent of the defects in CAKUT patients. To identify the developmental abnormalities that may cause the kidney and urinary tract malformations, we followed the formation of ureteric buds (UB), a key event that leads to the development of the renal collecting system and renal vesicles (8). In mice, UB

formation starts around embryonic day 10.5 (E10.5) in mice. At E11.0, a single UB is readily observed on each side in wild type embryos (Fig. 2A). We examined eleven *Gen1*$^{PB/PB}$ embryos at this stage (Table 1). Surprisingly, nine of the mutant embryos (81.8%) possessed an ectopic UB on one side (Fig. 2B). The others developed single UBs in a more anterior position to the end of the nephric duct on one or both sides (Fig. 2C). *Gen1*$^{PB/+}$ mice exhibit less severe defects, as only three of twenty-one (14.3%) *Gen1*$^{PB/+}$ embryos at E11.0 were detected to have an ectopic UB. Thus, disruption of *Gen1* leads to excessive UB formation in mice.

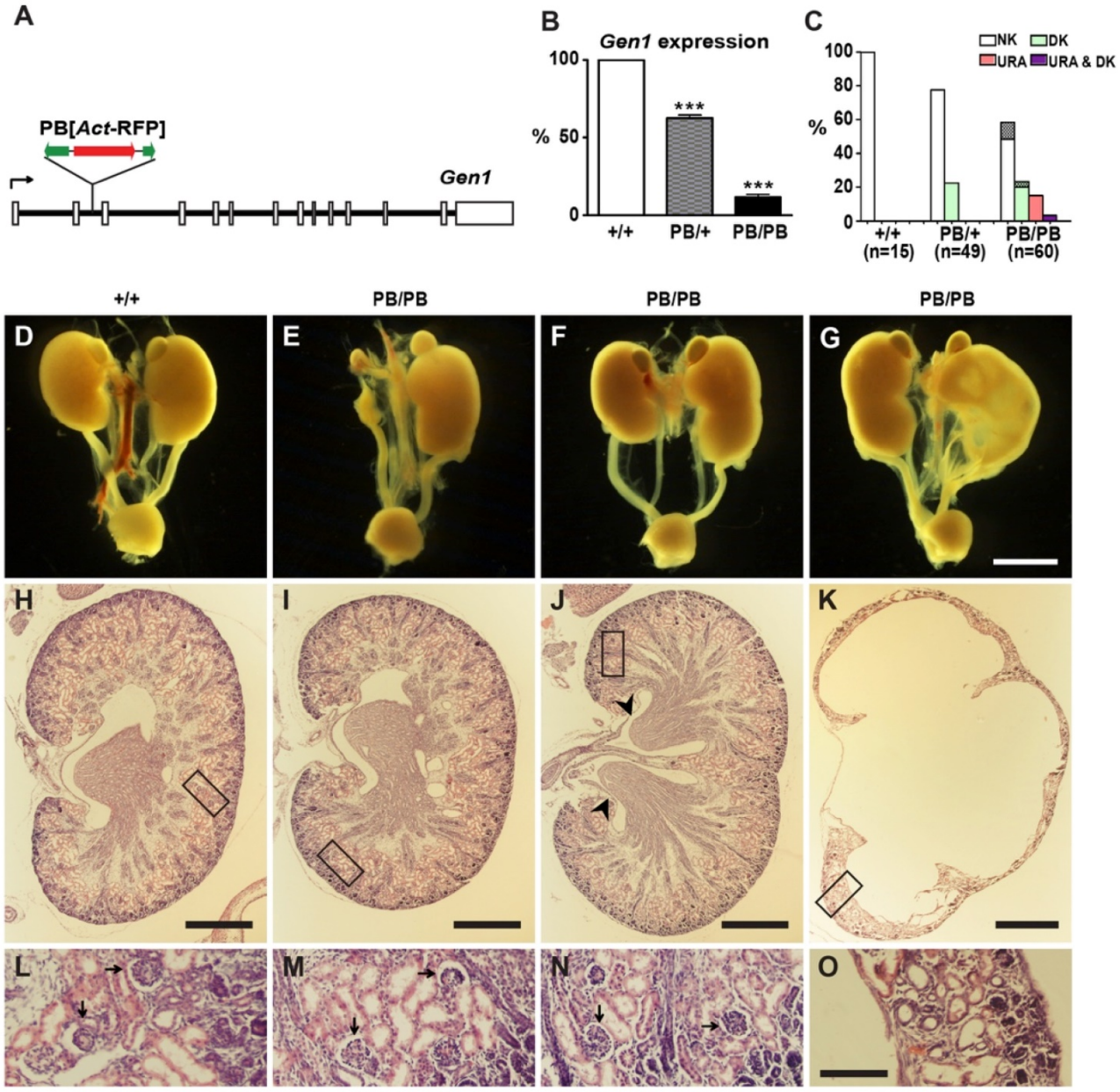

Figure 1. Kidney and urinary tract defects in *Gen1* mutants. (A) Genomic structure of the *Gen1*^{PB} allele. A PB[Act-RFP] transposon was inserted in the second intron (solid lines) of *Gen1*. Open boxes, exons; black arrow, transcription direction; green arrows, PB termini; red arrow, RFP expression cassette. (B) Real-time PCR analysis of *Gen1* expression in E12.5 embryos. Data is shown as the mean ± s.e.m. *** $P < 0.001$ determined by *t* test. (C) Percentages of newborn mice with normal number of kidneys (NK), unilateral renal agenesis (URA), or duplex kidneys (DK). Percentages of mice having accompanying hydronephrosis (HN) are indicated at the top of each column. (D-G) Representative images of the urinary system in newborn mice. URA (E), unilateral DK (F,G), severe HN and hydroureter (G) are presented. (H-O) Hematoxylin-eosin (H&E) staining of the paraffin sections of kidneys in newborn mice. A wild type kidney (H), URA (I), DK (J), and severe HN (K) are presented. Details within the boxes are enlarged in (L-O), respectively. DK is defined by double renal pelvises (arrowheads). Well differentiated glomeruli (arrows) could be observed in the wild type, URA, and DK. In contrast, tubular elements and glomeruli are almost missing in severe HN (O). Scale bars, 2 mm in D-G; 250 μm in H-K; 50 μm in L-O.

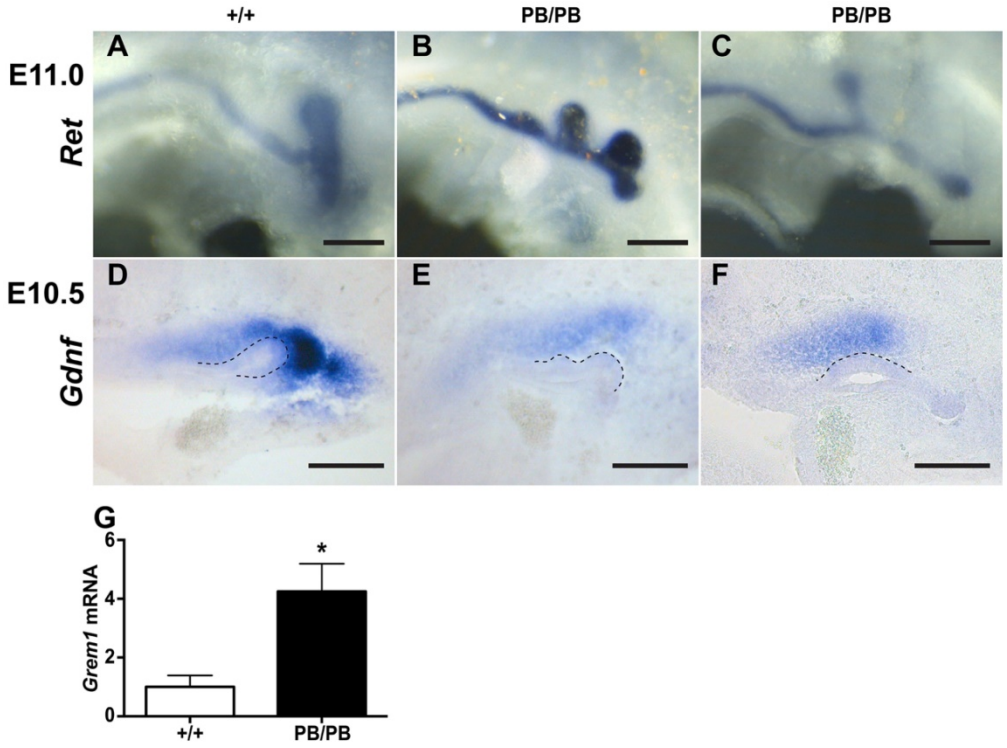

Figure 2. *Gen1* mutation leads to ectopic UBs accompanied by altered *Grem1* and *Gdnf* expression. (A-C) Ectopic UBs detected in E11 embryos by *c-Ret* RNA *in situ* hybridization. (D-F) *Gdnf* RNA *in situ* hybridization in E10.5 embryos. Homozygous embryos (E,F) express less *Gdnf* than the wild type (D). (G) Real-time RT-PCR of *Grem1* from E10.5 wild type (n=4) and homozygous mutant (n=6) kidney primordia. Dashed lines indicate UB. Scale bars, 0.2 mm. * $P<0.05$.

Table 1. Defective UB formation cases in E11 *Gen1* mutant embryos. The percentage of the embryos with indicated phenotype is shown in brackets. WT, wild type.

	Total embryos	Normal	Embryos with ectopic buds	Embryos with anterior buds
WT	8	8 (100%)	0	0
PB/+	21	18 (85.7%)	3 (14.3%)	0
PB/PB	11	0	9 (81.8%)	2 (19.2%)

Gen1 is ubiquitously expressed in the mesoderm of E10.5 embryos, including MM and UB regions (Supplementary Fig. 4). To explore if *Gen1* mutation alters the signals controlling UB formation, we examined the expression of genes belong to the GDNF, FGF, SLIT, and BMP pathways in E10.5 kidney primordia (Supplementary Table 1) (8, 9). Among them, the BMP4 antagonist *Grem1* is the only gene that was significantly upregulated, as revealed by RNA-seq. Real-time RT-PCR of E10.5 kidney primordia confirmed significant *Grem1* upregulation in $Gen1^{PB/PB}$ embryos (Fig. 2G). In addition to *Grem1*, RNA-seq revealed significantly lower expression of GDNF pathway genes *Eya1*, *Pax2*, *Pax8*, *Sall1*, *Six2*, and *Wt1* (Supplementary Table 1). We thus examined the expression of *Gdnf* in $Gen1^{PB}$ mutants by *in situ* hybridization, despite the fact that *Gdnf* expression alteration was not recognized by RNA-seq. We detected reduced *Gdnf* expression in six of eleven (54.5%) homozygous embryos (Fig. 2D-F).

Defective UB branching with decreased GNDF signal in *Gen1* mutants

Excessive UB formation usually leads to duplex kidneys. To explore the reason for frequently observed renal agenesis in *Gen1* mutants, we further examined the branching process after UB formation. In wild type mice, UB always branches in the metanephric mesenchyme (MM) to form a T-shaped bifurcation at E11.5 (Fig. 3A). In contrast, $Gen1^{PB/PB}$ embryos are often affected by UB branching failure at this stage (Table 2). In fact, we observed eleven branching failure events in forty-seven UBs from nineteen E11.5 homozygotes (Fig. 3B). Of eleven embryos without ectopic UBs, three had unilateral branching failures. In seven embryos with a unilateral ectopic UB, we observed three embryos with one unbranched UB and two with both on the side of two UBs, respectively (Fig. 3B). The only embryo with bilateral ectopic UBs also had branching failure in one of the UBs. UB branching defects were weaker in $Gen1^{PB/+}$ mice. In twenty-five E11.5 heterozygous embryos, no branching failure was observed in eighteen embryos without ectopic UB, while one out of seven embryos with a unilateral ectopic UB had one UB failed to branch on that side. These results suggest that UB branching failure as the reason for renal agenesis in *Gen1* mutants.

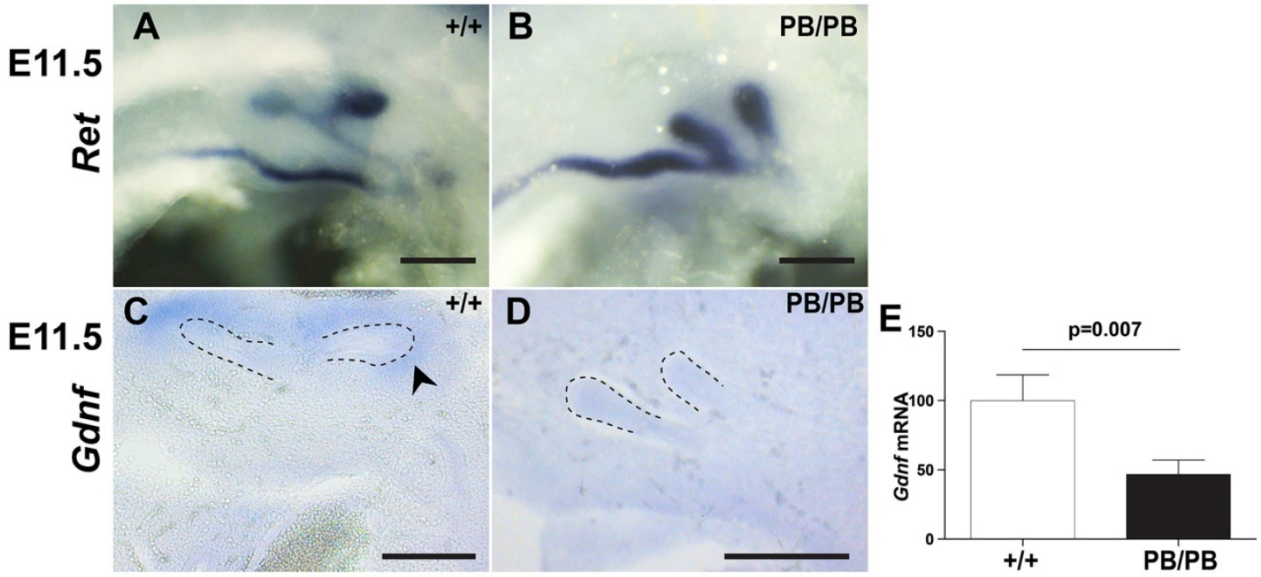

Figure 3. Ureteric branching defects in *Gen1* mutants. (A) *c-Ret* RNA in situ hybridization at E11.5 showed normal branch in wild type embryos. (B) Both UBs at the same side failed to branch in some E11.5 *Gen1*$^{PB/PB}$ embryos. (C) *Gdnf* RNA *in situ* hybridization showed *Gdnf* expression around the UB branches in E11.5 wild type embryos. (D) No *Gdnf* expression was detected around the un-branched UBs in E11.5 *Gen1*$^{PB/PB}$ embryos. (E) Quantitative RT-PCR showed decreased *Gdnf* expression in MM of E11.5 *Gen1*$^{PB/PB}$ embryos. Dashed lines indicate UB. Scale bars, 0.2 mm.

In wild type embryos, presence of GDNF in the condensed mesenchyme surrounding the tips of UB is essential for branching (Fig. 3C) (8). In E11.5 *Gen1*$^{PB/PB}$ embryos, *Gdnf* expression was present surrounding the tips of all thirty branched UBs but was undetectable in any of the seven unbranched UBs (Fig. 3D). Quantitative RT-PCR also detected approximately 50% drop of *Gdnf* transcription in *Gen1*$^{PB/PB}$ MM than that in wild type embryos (Fig. 3E). These results suggest that lower *Gdnf* expression caused by the *Gen1* mutation may critically contribute to UB branching defects in the mutants.

GEN1 binds with SIX1 and regulates the transcriptional activity of SIX1/EYA1 complex

We next explored the molecular mechanism through which *Gen1* regulates *Gdnf* during UB formation and branching. As a HJ resolvase, GEN1 was originally identified from nuclear extracts (24). It has been shown that approximately 20% of GEN1 proteins exist in the nuclear fraction during interphase (29). Since several transcription factors have been identified as regulators of *Gdnf* expression during early kidney development (12-14, 30-34), we hypothesize that GEN1 may interact with the upstream transcription factors to regulate *Gdnf* expression and tested potential interactions among them by Co-IP. We found that GEN1 could interact with SIX1, but not with its molecular partner EYA1, nor with SIX2, SIX4, PAX2, or FOXC1 in HEK293T cells (Fig. 4A, B and supplementary Fig. 5). Thus, GEN1 may interact with SIX1 to affect *Gdnf* expression.

SIX1 was proposed to form a functional complex with EYA1 in regulating *Gdnf* during early kidney development (9). We thus examined if GEN1 could alter the transcription activity of SIX1/EYA1 complex. Consensus SIX1 binding sites have been identified in the intron of *Gdnf* (35). We found the expression of a luciferase reporter driven by a minimal promoter and consensus SIX1 binding sites (Gdnf-luc2) remained at the basal level in HEK293T cells when GEN1 was provided alone. The presence of both SIX1 and EYA1 increased luciferase expression by about six times, while GEN1, SIX1 and EYA1 together enhanced the transcription by approximately twenty folds (Fig. 4C). Metanephric mesenchyme expression of *Six2* was also reduced in half (3/6) of the E10.5 *Gen1*$^{PB/PB}$ embryos (Fig. 4D, E). Taken together, these results suggested that GEN1 could enhance *Gdnf* expression by stimulating the transcriptional activity of the SIX1/EYA1 complex during early kidney development.

Table 2. Defective UB branching cases in E11.5 *Gen1* mutant embryos. The percentage of the embryos with indicated phenotype is shown in brackets. *, The only *Gen1*$^{PB/+}$ embryo with one unbranched UB has two buds on that side.

	Total embryos	Normal	Embryos with one unbranched UB	Embryos with two unbranched UBs
WT	14	14 (100%)	0	0
PB/+	25	24 (96%)	1 (4%)*	0
PB/PB	19	10 (52.6%)	7 (36.8%)	2 (10.5%)

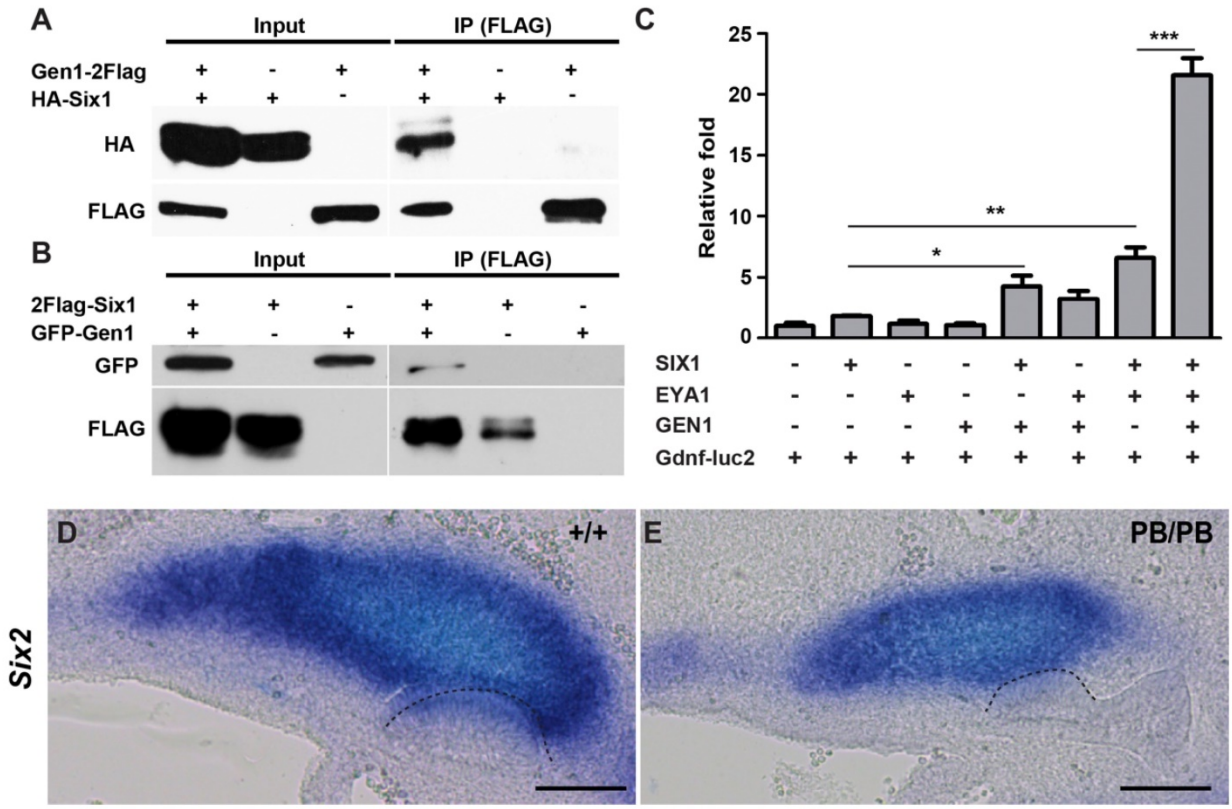

Figure 4. GEN1 binds with SIX1 and enhances transcriptional activity of the SIX1/EYA1 complex. (A) HA-tagged SIX1 can be pulled down by FLAG-tagged GEN1. (B) GFP-tagged GEN1 can be pulled down by FLAG-tagged SIX1. (C) Luciferase activity of a SIX1/EYA1 reporter. Supplemented by SIX1, EYA1, and GEN1, the luciferase became more active than under any other environment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by *t* test. (D,E) *In situ* hybridization at E10.5 revealed lower *Six2* expression in homozygous (E) than in wild type embryos (D). Dashed lines indicate UB. Full-length blots are presented in Supplementary Figure 6. Scale bar, 0.1 mm.

Previous studies have identified two conserved glutamic acid residues (E134/E136) required for GEN1 nuclease activity (24). We found the presence of a nuclease-inactive mutant, GEN1-2A (E134A/E136A), enhanced the expression of luciferase to similar levels as the wild type did (Fig. 5A, B). Thus, the resolvase activity of GEN1 is not required for GEN1 to promote SIX1/EYA1-mediated transcription. We also tested truncated GEN1 protein using the same assay. We found that a truncated GEN1 fragment (GEN1-N1, residues 1-585) that contains the potent resolvase fragment (24, 36) is able to enhance the activity of the SIX1/EYA1 complex, while two shorter fragments (GEN1-N2, residues 1-399; GEN1-N3, residues 1-319) could not (Fig. 5C). Co-IP experiment also proved impaired interaction between GEN1-N2/N3 and SIX1 (Fig. 5D). These data suggests that amino acids 400-585 are important for the transcriptional stimulation activity of GEN1.

Discussion

Initially purified as a mammalian Holliday junction resolvase, GEN1 was later reported necessary for centrosome integrity in mammalian cells (24-26, 29, 37). Here we found that *Gen1* is required for kidney and urinary tract development and neural tube closure in mice. These are the first evidence showing that *Gen1* is involved in developmental regulation. During UB formation, GEN1 not only affects the expression of BMP antagonist GREM1, but also stimulates the transcription of UB promoting signal GDNF, which may imply its role as a coordinator of both pathways to ensure normal nephrogenesis.

Intracellular localization was proposed as a critical factor for GEN1 activity. GEN1 was reported to be spatially regulated during cell cycle, such that its resolvase activity is restricted until mitosis when the nuclear membrane breaks down. With the strong nuclear exporting signal, it has been shown that approximately 80% of GEN1 proteins are presented in the cytoplasm (29). We have shown that GEN1 proteins could bind with the transcription factor SIX1 and enhance the transcriptional activity of the SIX1/EYA1 complex. Consistently, *Gen1* mutations led to decreased expression of SIX1 downstream genes during kidney development. These results suggest that the apparently minor nuclear fraction of GEN1 may be critical for developmental regulation.

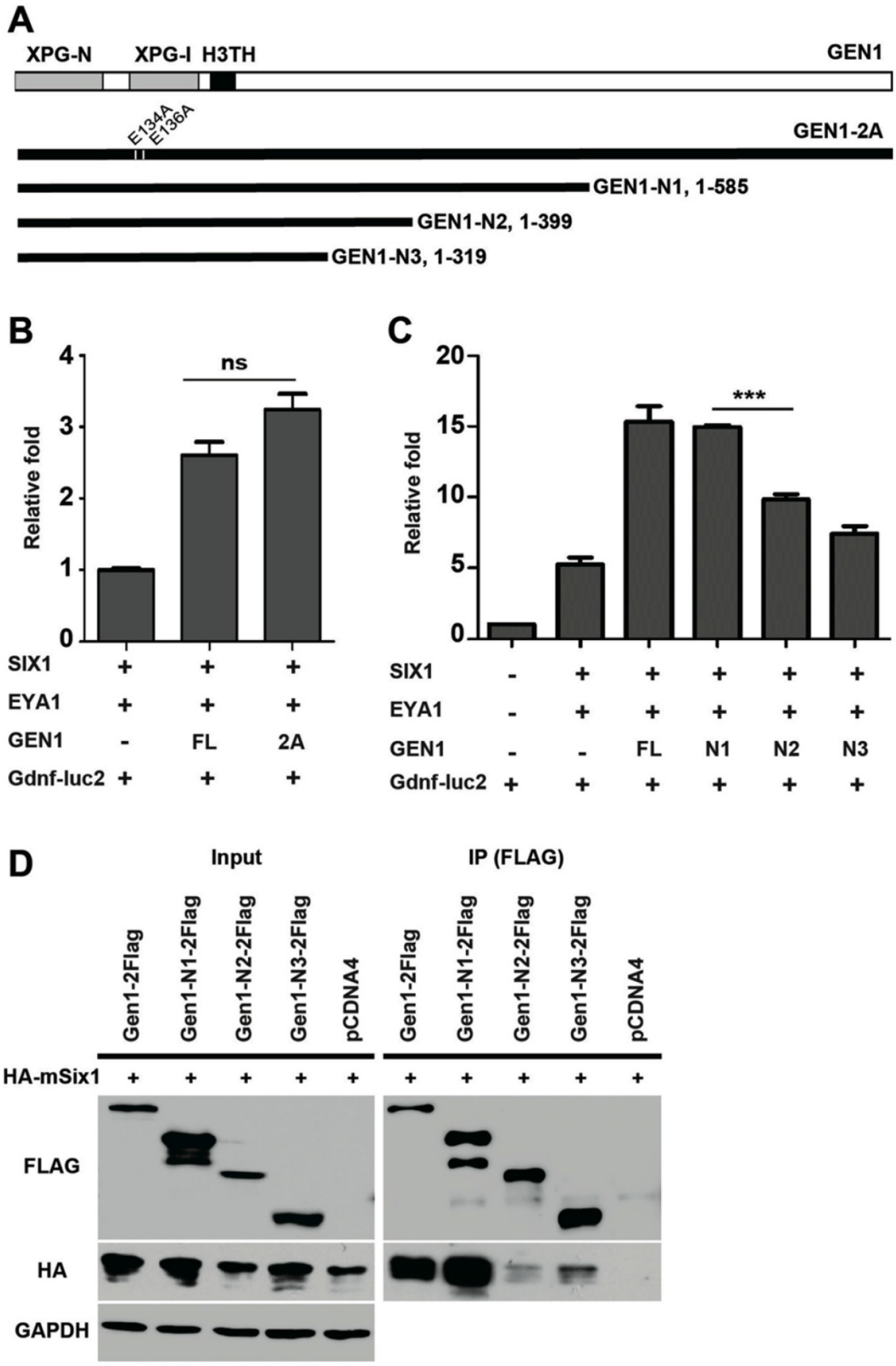

Figure 5. Impaired interaction between mutant GEN1 and SIX1. (A) Schematic view of mutant GEN1 proteins. (B) Luciferase assay showed potent activity of GEN1-2A in enhancing the transcriptional activity of SIX1/EYA1 complex. (C) Luciferase assay showed impaired activity of truncated GEN1s in enhancing the transcriptional activity of SIX1/EYA1 complex. (D) Co-IP experiments showed impaired binding activity between truncated GEN1s and SIX1. Full-length blots are presented in Supplementary Figure 7. XPG-N, N-terminal nuclease domain; XPG-I, internal nuclease domain; H3TH, helix-hairpin-helix DNA binding domain. FL, full length; 2A, E134A/E136A.

Not all *Gen1*$^{PB/PB}$ mice display kidney and urinary tract abnormalities. This may be due to the fact that *Gen1*^{PB} is a partial loss-of-function allele, such that significant amount of potent GEN1 still exist in the homozygous mutant mice. In fact, incomplete penetrance is not uncommon for mutations that affect kidney development. Heterozygotes of *Six1*, *Eya1*, or *Foxc1* knock out mutant mice all have varied kidney abnormalities (13, 15, 34). In addition, varied kidney and urinary tract abnormalities in *Gen1*$^{PB/PB}$ mice may reflect the complexity of the regulatory signals during early kidney development.

During UB formation, we have detected down-regulation of *Gdnf* signals but up-regulation of *Grem1*. *Gdnf* is usually considered as a UB promoting signal since complete ablation of *Gdnf* would lead to failed UB formation in mice (38). However, it needs to be pointed out that UB formation is sensitive to the dosage of *Gdnf* signals. In *Six1*$^{-/-}$ and *Sall1*$^{-/-}$ mutant embryos, *Gdnf* expression was partially decreased, which did not interfere with normal UB formation, but caused failure of the first UB branching (13, 39). On the other hand, GREM1-mediated BMP antagonism is essential to initiate UB formation (23). *Grem1* knockout mice exhibited disrupted metanephric development at the stage of UB formation; while additional recombinant GREM1 protein could induce additional budding of the wild type nephric duct (23, 40). In *Gen1*$^{PB/PB}$ mutants, *Gdnf* expression was partially reduced (Fig. 2E and F), while *Grem1* transcription was increased to four folds of wild type embryos (Fig. 2G). It is likely the decreased *Gdnf* signal did not affect UB formation in E10.5 *Gen1*$^{PB/PB}$ embryos, while the extra UB formation was due to the elevated *Grem1* expression. Thus, varied *Grem1* expression may lead to partially unobserved ectopic UB formation. During UB branching, ectopic UB in a single nephric duct may not always develop into a duplex kidney due to the lack of adequate GDNF signals so that less duplex kidney mutants were observed after birth. Unlike kidney defects, kinky tails were consistently observed in all *Gen1* homozygotes. This suggests that a different threshold of GEN1 activity or a different mechanism is required for neural tube development.

The trunk of the initial UB differentiates into ureter, connecting the kidney with the bladder (41). Higher or lower UB formation on the nephric duct causes abnormal positioning of the ureter orifice in the trigone, leading to obstruction or reflux (42). We have observed single UBs at more anterior position in two *Gen1*$^{PB/PB}$ embryos (Fig. 2C). These abnormally positioned ureters may join the bladder at a position more lateral or anterior to the proper site in the trigone. This could shorten the intra-vesicular ureteral segment and disrupt the valve mechanism that prevents the urine backflow to the kidney, thus resulted in VUR. On the other hand, although we didn't observe proximal ureteral atresia or PUJO in *Gen1* mutants (Fig.1), we could not exclude the possible contribution of potential ureteral atresia to the severe hydronephrotic phenotype. Similarly, our study did not exclude the possibility that *Gen1* mutation may cause delayed kidney development. To distinguish this event, global quantification of tissue dynamics can be applied to monitor the developmental process of nephron number and branching at different stages in *Gen1* mutant mice (43).

Our study raises the possibility of GEN1 as a disease gene of human CKAUT. To date, more than 90% of CAKUT patients still lack any molecular diagnosis (5-7). We have detected CAKUT phenotypes in both heterozygous and homozygous *Gen1* mutant mice and shown that GEN1 proteins containing the N-terminal 399 amino acids could not function properly in regulating the transcriptional activity of SIX1/EYA1. At the same time, thirty-seven loss-of-function variants have been reported in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org), all of which are presented in heterozygosity and predicted to encode truncated GEN1 proteins with less than 400 residues in the N-terminus. Although incomplete penetrance, as implied by the mouse data, may bring complications in human genetic analysis, it is still worth to explore the genetic burden of *GEN1* mutations in CAKUT patients.

Methods

Mice

All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine (IDM) at Fudan University. The *Gen1* mutant strain (081125049-HLA) was established and maintained on the FVB/N background. In the *Gen1*^{PB} allele, the *PB* insertion was mapped in the second intron of *Gen1* (Chr:12.11268138, Ensembl release 54).

PCR

Genotyping PCR was performed with a *PB* specific primer LB2 (5'-CTGAGATGTCCTAAATGCA CAGCG-3') and two flanking genomic primers 081125049-L1A (5'-TAGTGTGGGCACATGCAAGC-3') and 081125049-R1A (5'-CCCCTTTGCCTGTTCTTA ACCTC-3'). Real-time RT PCR was performed with SYBR Green (Agilent) according to the manufacturer's instruction. *Gapdh* was selected as the internal control

for normalization. The primers used for RT-PCR were: Gen1-RT-F1 in exon 2: 5′-GCACAGACAGTGAAGAAAATG-3′; Gen1-RT-B1 in exon 3: 5′-TGCTTATGACATCAGCTTTCAG-3′; PCR product size 151 bp. Grem1-RT-F: 5′-CCTTTCAGTCTTGCTCCTTCTGC-3′; Grem1-RT-R: 5′-TTCTTCTTGGTGGGTGGCTGTAGC-3′; PCR product size 94 bp. Gapdh-RT-F1: 5′-TGTTCCTACCCCCAATGTGTCC-3′; Gaphd-RT-B1: 5′-GGAGTTGCTGTTGAAGTCGCAG-3′. PCR product size 169 bp.

Histology

Kidneys and the urinary tract were dissected and embedded with paraffin according to the standard protocol (31). Hematoxylin-eosin (H&E) staining was then performed on the sections of 7 μm as previously described (31).

RNA-seq

Total RNA from kidney primordia of E10.5 embryos was extracted by TRIzol (Invitrogen) and GlycoBlue (Ambion) coprecipitation. We combined two mice to obtain 0.5 μg of total RNA for either wild type or *Gen1*$^{PB/PB}$ samples. Strand-specific RNA-seq libraries starting with 0.5 μg of total RNA were constructed as previously described (44). Two biological replicates of RNA-seq for each group (wild type or mutants) were included to analyze differentially expressed genes. The RNA-seq libraries were sequenced on the Illumina HiSeq2000 platform (Genergy Biotechnology, Shanghai). FastQC was used to evaluate the quality of high-throughput sequencing data (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Paired-end reads were mapped to mm9 genome using TopHat2 with default parameters except –g –r and –mate-std-dev (45). In specificity, -g was set to 1 to ensure that there is only one alignment for a given read (45). Bowtie was used to get a good approximation of –r and –mate-std-dev, which is the expected inner distance between mate pairs and the standard deviation for the distribution on inner distances between mate pairs, respectively (46). The known transcripts supplied to TopHat2 were based on the Ensemble database and the NONCODE database (47). Cufflinks was used to assemble transcripts and estimates their abundances for each sample and Cuffdiff was used to get the differentially expressed genes (48).

RNA *in situ* hybridization

The coding sequences of *Ret, Gdnf,* and *Six2* were cloned from E12.5 embryos through RT-PCR. Digoxigenin-labeled anti-sense probes were then produced by the DIG RNA Labeling kit (Sp6/T7) (Roche). Whole-mount *in situ* hybridization was performed as previously described (49). Frozen sections of 50 μm were then used for signal detection.

Co-immunoprecipitation (Co-IP) and luciferase assay

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen) for both co-immunoprecipitation and luciferase assay. Co-IP and immunoblot were performed as previously described (50). The antibodies used include mouse anti-HA (Covance), rabbit anti-HA (Abcam), mouse anti-FLAG (Sigma), and rabbit-anti-GFP (Cell signaling). For luciferase assay, each transfection was triplicated with 400 ng wild type or mutant *Gen1*, 100 ng pCX-EGFP, 200 ng HA-Six1, 200 ng HA-Eya1, and 200 ng Gdnf-luc2. Luciferase activity was scored 48 hours after transfection and calibrated by the percentage of GFP positive cells (determined by FACS). Each experiment was performed at least twice independently.

Statistics

All data are presented as mean ± SEM. The comparison of two groups was performed with unpaired two-tailed *t*-test. The threshold for significance was set at $P < 0.05$.

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

Supplementary Material

Supplementary figures and tables.
http://www.ijbs.com/v14p0010s1.pdf

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Author Contributions

H.W. and C.Z. designed and performed experiments, interpreted data, and wrote the manuscript. X.W. (Xiaowen Wang) performed histology analysis. B.G., Z.G., Y.B., Q.S., X.W. (Xiang Wang), and J.L. participated in phenotypic analysis. Y.L., M.H., and T.N. carried out RNA-seq analysis. X.Z. (Xiaoe Zhang), X.Z. (Xiaoting Zhu) and S.X. participated in molecular biology experiments. Y.Z. gave intellectual input; H.X. supervised the collaborations. X.W. (Xiaohui Wu) conceived of and designed the studies, supervised the work, and wrote the manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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