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Sox11 gene disruption causes congenital anomalies of the kidney and urinary tract (CAKUT)

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Abstract

Congenital abnormalities of the kidney and the urinary tract (CAKUT) belong to the most common birth defects in human, but the molecular basis for the majority of CAKUT patients remains unknown. Here we show that the transcription factor SOX11 is a crucial regulator of kidney development. SOX11 is expressed in both mesenchymal and epithelial components of the early kidney anlagen. Deletion of *Sox11* in mice causes an extension of the domain expressing *Gdnf* within rostral regions of the nephrogenic cord and results in duplex kidney formation. On the molecular level SOX11 directly binds and regulates a locus control region of the protocadherin B cluster. At later stages of kidney development, SOX11 becomes restricted to the intermediate

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DISCLOSURE

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segment of the developing nephron where it is required for the elongation of Henle's loop. Finally, mutation analysis in a cohort of patients suffering from CAKUT identified a series of rare *SOX11* variants, one of which interferes with the transactivation capacity of the SOX11 protein. Taken together these data demonstrate a key role for SOX11 in normal kidney development and may suggest that variants in this gene predispose to CAKUT in humans.

Keywords

CAKUT; duplex kidneys; kidney induction; nephron; Sox11

With an incidence of 1 in 500, congenital abnormalities of the kidney and urinary tract (CAKUT) belong to the most common defects in the unborn child. CAKUT unites a diverse group of disease entities including renal agenesis, hypoplasia and dysplasia, as well as defects affecting the ureter.[1] Despite its importance as the major cause of renal failure in children, the molecular basis of CAKUT is unknown in as many as 80% of cases.[2]

CAKUT is rooted in defective kidney development. In the mouse embryo, metanephric development commences at around embryonic day (E)10 with the specification of the metanephric mesenchyme (MM) at the caudal end of the nephrogenic cord.[3] MM cells release the glial-derived neurotrophic factor (GDNF), which serves as an inductive signal that initiates ureteric budding from the Wolffian duct. At the molecular level, GDNF binds at the surface of Wolffian duct cells to its tyrosine kinase receptor RET and induces ureteric bud (UB) growth and branching.[4] The UB subsequently invades the MM to undergo a series of dichotomous branching events giving rise to the ureteric tree. Induction of the UB outgrowth must be tightly controlled, as failure of UB induction leads to renal agenesis.[5] Conversely, ectopic signaling causes supernumerary UBs that can lead to duplex kidneys, a condition that is frequently associated with hydronephrosis and hydroureter.[6,7]

Signals released from the ureter induce mesenchymal cells to epithelialize through the mesenchymal to epithelial transition,[8] and the resulting renal vesicle undergoes further patterning events and morphogenetic movements to form the differentiated nephron, the functional unit of the kidney. Nephrons are highly segmented, with the glomerulus, proximal tubule, Henle's loop, and distal tubule serving distinct functions and expressing a specific set of marker genes.

The *Sox* family comprises 20 genes that encode transcription factors involved in a diverse range of developmental and pathologic processes.[9] Based on the phylogenetic homology of their HMG box DNA-binding domain, *Sox* genes are categorized into 8 groups.[10] We previously reported an important function of *Sox8* and *Sox9* in kidney development[11] and renal repair.[12] Members of the *SoxC* group, *Sox4, Sox11*, and *Sox12*, are also expressed in kidney development, and *Sox4* has been suggested to play a role in nephron endowment.[13] *Sox11* knockout analysis has revealed its requirement for the development of a variety of organs,[14,15] but potential involvement of this gene in kidney development has not yet been reported.

Here we show that SOX11 fulfills essential regulatory functions during early kidney induction, as well as nephron maturation in mice. Moreover, the identification and characterization of rare human *SOX11* gene variants in CAKUT patients further suggest that this gene may also contribute to human kidney disease.

RESULTS

SOX11 is required for normal development of the urogenital tract

Although previous studies have already reported *Sox11* to be expressed in kidney development,[13,14,16] a detailed analysis, in particular at early stages of kidney induction, has been missing. Immunostaining at E11 revealed relatively broad SOX11 expression in the mesenchyme of the nephrogenic cord, the UB, and the Wolffian duct (Figure 1a and Supplementary Figure S1). At later stages, SOX11 expression was maintained in the epithelial and mesenchymal compartments (ureter, UB, and cap mesenchyme, respectively) of the developing kidney (Figure 1a), but was absent in FoxD1-positive stromal cells (Figure 1b). Upon mesenchymal to epithelial transition, robust staining was observed throughout the newly formed renal vesicle, which, by the S-shaped body stage, was strongest within the intermediate segment (future Henle's loop) (Figure 1c). Upon differentiation, expression persisted at low levels in elongating nephron tubules (Figure 1a, right panel), but no signal was detected in fully differentiated cells or adult kidneys (Figure 1d). Similar to kidney development, *Sox11* was found broadly expressed at early stages (E12.5) of urethra development, but was no longer detectable from E14.5 onward (Figure 1e and data not shown).

To analyze a potential role of SOX11 in urogenital development, we used a previously reported knockout allele that in the homozygous state results in perinatal death due to heart failure.[14] Analysis of dissected urinary tracts in E18.5 embryos revealed a range of defects including unilateral and bilateral duplex kidneys, hydronephrosis, hydroureter, and abnormally positioned ureters (Figure 2, Table 1, and data not shown). Interestingly, duplex kidneys were also seen in a small proportion of heterozygous mutants (5.1%), indicating *Sox11* haploinsufficiency (Figure 2b, e, i and Table 1). Histologic analysis revealed that duplex kidneys were associated with an ectopic ureter that connects to the bladder in a position caudal to that of the normal site (Figure 2h). In addition to kidney and ureter defects, a proportion of *Sox11* null mutants also displayed genital tract defects, including misplaced ovaries and undescended testes (Supplementary Figure S2). When present, these defects were always found in association with duplex kidneys, suggesting that the abnormal position of the gonads might be linked to renal abnormalities.

Sox11 restricts ureter budding to a single site

The occurrence of duplex kidneys in *Sox11* mutants suggested an early developmental defect. Indeed, dissection of kidney rudiments at E11.5 revealed 2 UBs that, when cultured, developed into 2 kidneys (Supplementary Figure S3). Ureteric budding is primarily regulated by GDNF/Ret signaling.[3] To test whether ectopic budding may be caused by a disruption of this signaling pathway, we performed quantitative polymerase chain reaction (PCR) and whole-mount *in situ* hybridization (ISH) analysis on E10.5 and 11.5 embryos.

qPCR analysis of *Ret* and *Gdnf* expression did not reveal any significant change in expression levels in wild-type and mutant embryos (Supplementary Figure S4A and B). As expected, *Ret* expression was found within the mesonephric duct and the T-shaped branched ureter in wild-type embryos (Figure 3a). In homozygous *Sox11* mutants, the overall *Ret* expression pattern was unchanged, although it highlighted the second UB emerging slightly rostrally to the normal induction site (Figure 3b).

In contrast to the epithelial *Ret* expression, *Gdnf* is expressed in mesenchymal renal progenitor cells. In wild-type mice, low levels of *Gdnf* can be found as early as E9.5 along the entire length of the mesonephric mesenchyme, but by E10.75, expression becomes restricted to the MM at the caudal end of the nephrogenic cord (for a review, see Davis *et al.*[5]). Strikingly, homozygous mutants displayed a dramatic rostral extension of the *Gdnf* expression domain when compared with wild-type embryos of the same stage (Figure 3c and d and Supplementary Figure S4C and D). More anterior staining was also seen when using other MM-specific markers such as *Six2* (Figure 3e and f), indicating a rostral expansion of MM cells rather than a simple upregulation of *Gdnf* in *Sox11* mutant embryos. Taken together, these data show that SOX11 is required to restrict *Gdnf*-expressing cells to the caudal end of the nephrogenic cord.

SOX11 regulates the protocadherin β genes by directly binding to its cluster control region

SOX11 is foremost a transcriptional regulator, and loss of function is likely to affect the expression of downstream target genes. Formation of ectopic UBs has been reported previously in mouse mutants including *Gata3*,[17] *Sprouty1*,[18] *Bmp4*,[19] *Foxc1/2*, [7] *Robo2*, and its receptor *Slit*.[6] ISH expression analysis showed no significant changes in these genes (Supplementary Figure S5 and data not shown), suggesting that SOX11 acts through independent molecular pathways.

To obtain an unbiased view on genes whose expression depends on SOX11, we performed microarray analysis on RNA isolated from the caudal region of E10.75 wild-type and mutant nephrogenic cords. A total of 281 probe sets presented statistically significant changes (76 up, 205 down; false discovery rate <0.1) with 68 of these (13 up and 55 down), showing >1.5 fold up-/downregulation (Supplementary Data 1). Expression of genes known to be involved in duplex kidney formation was not significantly changed in mutant mice, thus further corroborating our *in situ* hybridization analysis.

Bioinformatic analysis using the DAVID tool[20] revealed a 144-fold enrichment of the protocadherin β annotation cluster ($P = 6.3e\text{-}18$) (Supplementary Data 2) with 17 of 22 genes being downregulated in *Sox11* mutants (Figure 4a). Protocadherin β genes are organized in a cluster on mouse chromosome 18,[21] and mapping the fold-change of probe sets along the chromosome showed a compelling reduction of expression of the entire gene family (Figure 4b). Previous analysis had identified a cluster control region mapping 320 kb downstream of the protocadherin β locus that seems to be required for the expression of the 22 *Pcdhb* genes.[22] To test whether SOX11 might directly bind the cluster control region, we performed chromatin immunoprecipitation quantitative polymerase chain reaction experiments on E14.5 wild-type kidneys. Strikingly, an 8-fold enrichment of chromatin precipitated with SOX11 (over IgG) was found at hypersensitive site HS19-20

of the cluster control region (Figure 4c). No such enrichment could be detected with primers for a negative control region, located in the 3′UTR region of the *Cd24a* gene. We concluded that direct binding of SOX11 to the locus control region is essential to provide high levels of expression of the *PcdhB* gene cluster.

Nephron segment defects in *Sox11* mutants

The dynamic expression pattern of *Sox11* during nephron formation prompted us to analyze a potential function of this gene at later stages of kidney development. Examination of E18.5 kidney sections revealed hypodysplastic kidneys with an apparent reduction of epithelial components and an increase of stromal tissue in the medulla (Figure 5a–b'). A proportion of mice also displayed mild proximal tubule dilation, but this phenotype was not 100% penetrant (data not shown). ISH experiments revealed persistent expression of proximal (*Slc5a2* and *Slc5a1*), distal, and connecting tubule ((*Slc12a3* [*Ncc*])) markers, but dramatically shortened Henle's loops (*Slc12a1* [*Nkcc2*]) (Figure 5c–j), which was also reflected by reduced expression of *Irx1* and *Irx2* (Supplementary Figure S6A–D). To obtain a more quantitative evaluation of segment specific changes, we next counted the number of structures/cells highlighted by specific markers. Glomerular numbers, as defined by the expression of podocin (NPHS2), were unchanged in mutant kidneys, indicating that loss of *Sox11* does not interfere with nephron induction and glomerular maturation (Figure 6a–c). Immunostaining with antibodies detecting lotus tetragonolobus lectin (LTL) (proximal tubule) showed a small decrease, which, however, did not reach statistical significance (Figure 6d–f). By contrast, the number of cells positive for markers identifying Henle's loops (Tamm-Horsfall protein) and, to a lesser extent, the distal convoluted tubule (NaCl cotransporter) were significantly reduced, indicating a particular need for SOX11 for these segments (Figure 6g–l). Interestingly, number of cells positive for Henle's loop and distal tubule markers was also reduced in *Sox11* heterozygous mutants, suggesting that SOX11 acts in a dose-sensitive manner also during nephron maturation. Analysis of early nephron patterning markers (WT1, PAX2, JAG1, HNF1β, BRN1, SOX9, and *Papss2*) revealed normal expression (Supplementary Figure S6E–H).

Previous studies have shown that elongation of Henle's loops depends on collecting duct–derived *Wnt7b* that induces canonical β-catenin signaling in surrounding stromal cells, resulting in the activation of *Lef1*.[23] qPCR analyses revealed a mild increase of the Wnt inhibitor *Dkk1* in *Sox11* mutant (Supplementary Figure S7C). However, ISH, immunofluorescence, and qPCR analyses did not reveal significant changes in *Wnt7b, Lef1*, and *Axin2* expression, thus ruling out regulation of this pathway by SOX11 (Supplementary Figure S7C and D). Moreover, mitotic figures (phosphohistone H3) revealed no significant changes in the proliferation rate of early nephron structures nor in elongating loops of Henle (Supplementary Figure S7A and B). Similarly, apoptotic figures were unchanged between wild type and controls (data not shown).

Analysis of *SOX11* in a cohort of human CAKUT patients

Urogenital tract defects observed in *Sox11* null mice are reminiscent of congenital abnormalities observed in human CAKUT patients. To determine whether SOX11 variants are associated with CAKUT, we screened the coding sequence of *SOX11* (NM_003108.3)

in a cohort of 560 patients (Supplementary Table S1). A total of 6 heterozygous variants were detected in 6 patients, including 2 silent point mutations, 3 missense point mutations, and 1 insertion of 12 nucleotides, resulting in an in-frame insertion of 4 amino acids (Figure 7). One patient (patient 5) with posterior urethral valves and kidney hypoplasia showed 2 *SOX11* variants, including a 12-bp insertion and a missense mutation.

Comparison of our variants with online databases (1000 Genomes Project,[24] NCBI database of genetic variation dbSNP,[25] and ExAC Browser[26]) revealed the presence of 4 variants (c.63G>T, c.257G>T, c.658G>A, and c.1131C>T [not validated]) in previously sequenced populations (Table 2). The other 2 variants (c.995C>T and c.1063_1064insAGCGGCAGCAGC) were absent from public databases. Online mutation prediction using RegRNA 2.0 software[27] suggested that the silent C.1131C>T variant may introduce a novel splice donor site (Supplementary Data File 3). SIFT analysis (http://sift.jcvi.org/) did not reach a high enough score, but both PolyPhen-2 (genetics.bwh.harvard.edu/pph2) and Mutation tasting (http://www.mutationtaster.org/) considered several variants as damaging. Of particular interest is variant c.257G>T (p.R86L), as it affects the HMG box DNA binding domain, which has been highly conserved throughout evolution.

SoxC class proteins can act as classic transcription factors that bind to DNA and activate downstream target genes. To test whether the variants would interfere with transactivation, we recreated expression vectors and cotransfected them with a luciferase reporter plasmid of the *Gdf5* promoter, a proposed downstream target of SOX11.[28] Most variants had no dramatic effect on reporter gene expression (Supplementary Figure S8). One exception was the insertion mutation c.1063_1064insAGCGGCAGCAGC, which completely abrogated reporter plasmid activation.

DISCUSSION

Kidney development is a highly complex process that requires the orchestrated action of transcription factors to ensure proper induction, proliferation, and differentiation. SOX genes are one class of factors that have already previously been implicated in kidney development. Although SOX8 and SOX9 are required for ureter branching,[11] SOX4 has been suggested to be required for nephron endowment.[13] Here we identified SOX11 as a novel player in kidney formation that appears to act during metanephros induction and nephrogenesis.

The first step in kidney development involves specification of the MM within the nephrogenic cord, with *Gdnf* and *Six2* often being used as molecular markers for this compartment. Both of these genes are expressed in *Sox11* mutants, indicating that specification occurs normally in the absence of this gene. Ureter induction relies on GDNF/rearranged during transfection signaling, and alterations in this pathway can lead to renal agenesis or ectopic budding. Overall expression levels of *Ret* and *Gdnf* appeared unaffected in mutants when analyzed by qPCR. ISH analysis, however, revealed a dramatic rostral extension of the *Gdnf* expression domain, which could be explained by several mechanisms: (i) A requirement for SOX11 to suppress *Gdnf* expression; this seems unlikely as SOX11 expression is also found in MM and thus overlaps with *Gdnf* and *Six2*; (ii)

a lack of apoptosis in rostral domains; (iii) disrupted cell migration of rostrally located MM precursors toward the caudal end. Although directed migration of mesenchymal cells toward the caudal end of the intermediate mesoderm has not been demonstrated so far, 2 previous studies using *Wnt5a/Ror2* knockout mice have suggested that such a migration may occur.[29,30] Several lines of evidence from our own studies further support this hypothesis. First, the extended expression domain in the rostral mesonephros is accompanied by a thinning of the *Gdnf*$^{+}$ and *Six2*$^{+}$ domain in the caudal region (Figure 3), an observation that is consistent with a disrupted migration of MM cells. Second, our microarray analysis in E10.75 *Sox11* mutants revealed a dramatic loss of molecules involved in cell adhesion and cell migration, such as the protocadherin-β cluster and members of the semaphorin signaling pathway (*Sema6C, Dpysl5*). Although duplex formation in mutant mice for these genes[31,32] nor the *Pcdhb* locus control region[22] has not been reported, mutations in the semaphorin family member *Sema3a* leads to increased ureter branching.[33] We therefore hypothesize that the combined reduction of these genes causes defects in cell adhesion and migration. A role for SOX11 as a regulator of cellular migration was previously shown in mesenchymal stem cells.[34] Further experiments will need to be performed to demonstrate active migration of *Gdnf*-positive mesenchymal cells toward the caudal end of the nephrogenic cord as a potential mechanism required for the formation of a bona fide metanephric mesenchyme. If our model turns out to be correct, mesenchymal cell migration would mirror the active migration of epithelial cells within the mesonephric duct that has been shown previously to be essential for proper kidney induction.[35,36]

Although expression of SOX11 is maintained in the ureter and MM throughout development, deletion does not seem to affect ureter branching, and based on glomerular counts, *Sox11* mutants develop the normal number of nephrons. This finding is somewhat surprising given that previous studies have suggested an important function for SOX11 in regulating *Wnt4* during nephron formation at least in *Xenopus* embryos.[37] The persistent nephrogenesis could potentially be explained through functional redundancy with other members of the SOXC gene family (SOX4 and SOX12). By contrast, *Sox11* seems to have a unique role during nephron formation, and mutants display a dramatically shortened Henle's loop, whereas other segments were only mildly affected. Nephron patterning occurs at a very early stage during morphogenesis, and several key genes have been identified in recent years.[38,39] Molecular analysis revealed the expression of all segment specific markers, indicating that initial patterning of the nephron occurs in *Sox11* mutant mice. Assignment of segment identity involves a careful balance between β-catenin, Notch, and BMP signaling, with distal/intermediate segments showing the highest β-catenin activity.[38] Because the proliferation rate in both S-shaped bodies and elongating Henle's loops was unchanged in mutants, we can speculate that the initial number of progenitors within the intermediate segments may be reduced. Proving this hypothesis would require a careful 3-dimensional analysis of a precisely defined nephron stage, which is difficult due to the highly dynamic nature of nephrogenesis.

During fetal life, gonads are anchored in the abdominal wall by the cranial suspensory ligament and the gubernaculum, at their cranial and caudal poles, respectively. Sex dimorphic regression/development of both cranial suspensory ligament/gubernaculum causes testicular descent, whereas ovaries stay localized at the lower poles of the kidneys,

processes controlled by testicular hormones.[40–42] Given the fact that the position of both testes and ovaries is affected in *Sox11* mutant mice, the absence of male hormones appears unlikely to be the cause of these defects. Alternatively, *Sox11* may have a hormone-independent role within cranial suspensory ligament and/or gubernaculum to allow their proper morphogenesis. A third possible explanation involves interdependence of urinary and genital tract defects. Several reports have shown abnormal localization of the gonads in mouse mutants with ureter malformations[7,18,43] with failure of gonadal duct/ureter separation due to common nephric duct persistence. Interestingly, ectopic testes in *Sox11*$^{-/-}$ mice are associated with a hydroureter connecting to the vas deferens (Supplementary Figure S2D'). Future work investigating the roles of *Sox11* in the development of gonads, cranial suspensory ligament/gubernaculum, and the common nephric duct will be needed to identify in which compartment this gene is required for proper genital tract development.

The incomplete penetrance of the phenotype is in line with observations made for CAKUT in humans. Indeed, disease-causing mutations are often transmitted from phenotypically normal parents to their children, thus highlighting the complexity of the genetics and a potential contribution of stochastic and environmental factors in complex diseases like CAKUT.[44] The fact that defects could be observed in a proportion of heterozygote mice supports gene dose sensitivity and is in line with the type of genetic alteration associated with CAKUT in human patients. The broad range of urogenital defects observed in *Sox11* mutant mice also corresponds to the observations of diverse phenotypes made in familial CAKUT.

SOX11 gene mutation analysis revealed a 12-bp insertion and 5 point mutations in a large cohort of CAKUT patients. These patients display different types of urogenital tract malformations that are recapitulated in the *Sox11* mouse model, namely, duplex kidneys, renal hypoplasia, testis, and ureteropelvic junction obstruction. Strikingly, 4 of the 5 male patients have ureter malformations. In mice, SOX11 is expressed in early urogenital sinus, which correlates with its potential role in ureteral development.

Although the pathogenic significance of the identified variants is uncertain at present, the complete absence or very low frequency of these genetic variants in public databases, further supported by *in silico* predictions, may suggest that at least some of them could be involved in the development of CAKUT. Functional analysis in transfection assays confirmed the disruptive nature of 1 variant for transcriptional activity of SOX11. Of interest, this variant has been recently identified in a patient with coloboma,[45] thus further implicating an important role for SOX11 disruption in human disease. It is challenging to determine *in vitro* whether the other variants identified in this study contribute to disease etiology. Modeling the mutations *in vivo* using CRISPR/Cas9 approaches may address this problem.

In conclusion, our study identified SOX11 as a crucial regulator of kidney development. SOX11 restricts *Gdnf*-expressing cells to the caudal part of the nephrogenic cord, controls single UB outgrowth, and is required for proper nephron formation. Although our study does not unequivocally prove an involvement of SOX11 in human CAKUT, the identification of rare variants in patients in combination with the phenotype observed in our mouse model makes it a strong candidate for human CAKUT disease.

MATERIALS AND METHODS

Mice

All animal work was conducted according to national and international guidelines and was approved by the local ethics committee (NCE/2011-19). The *Sox11* knockout mouse strain was described previously.[14] *Sox11* knockout mice were maintained on a mixed B6D2F1 genetic background. All comparisons were done between littermates of the same strains. Routine genotyping of mice was carried out by PCR, as previously described.[11]

Glomeruli and cell number quantification

Paraffin sections of 6 independent E17.5 embryos for each condition (control, heterozygote, and *Sox11* knockout) were processed in immunofluorescence with antibodies against WT1 and podocin/NPHS2 or WT1 and Nephrin/NPHS1. For segment-specific cell number quantification, paraffin sections of 3 independent E17.5 embryos from the 3 different conditions were stained for LTL, Tamm-Horsfall protein, or NaCl cotransporter. Photographs were taken with an Axiocam Mrm camera (Carl Zeiss AC, Oberkochen, Germany) and processed with ImageJ. Glomeruli and cells were counted manually by a single blind operator. Statistical analysis was performed using Student *t* test.

Proliferation assay

For elongating Henle's loop analysis, mice were injected with bromodeoxyuridine i.p. on day 17.5 of pregnancy and killed 90 minutes later. Embryo sections were stained for bromodeoxyuridine, BRN1, LTL, and lectin Dolichos biflorus agglutinin (DBA), and cells were counted using the Cell Counter plugin in Fiji software. The total number of bromodeoxyuridine-positive/$BRN1^+$/LTL^-/DBA^- cells was normalized on the total number of $BRN1^+$/LTL^-/DBA^- cells and expressed as a percentage.

For early nephron analysis, E16.5 embryo sections were stained for pHH3 (cellular mitotic bodies), JAG1, and WT1. Transverse sections of renal vesicles, comma-shaped bodies, and S-shaped bodies were counted by a single blinded observer using the Cell Counter plugin in the Fiji software. A section of early nephron structure was considered positive if it contained ≥1 mitotic figures positive for pHH3. S-shaped bodies were divided into a medial segment ($JAG1^+$) and a distal segment ($JAG1^-$). The total number of positive early nephron sections was normalized on the total number of early nephron sections and expressed as a percentage.

Data were obtained from 3 biological replicates for each condition. $Sox11^{+/-}$ and $Sox11^{+/+}$ samples were considered as controls for this experiment. Statistical significance of the results was evaluated with the Student *t* test.

Microarray study and data processing

Total RNA was extracted in 4 independent replicates from a dissected caudal area of urogenital ridges of *Sox11* knockout and wild-type E10.75 embryos using the RNeasy MicroKit (Qiagen, Hilden, Germany) followed by column DNase digestion to remove any contaminating genomic DNA. RNA preparations from the 2 different conditions were processed and hybridized on the Mouse Gene Array (Thermo Fisher Scientific, Waltham,

MA). Arrays were quantile normalized with respect to the probe GC content using the RMA algorithm. No or low expressed transcripts were removed by a maximum expression cutoff <50. The data filtering in 206768 of 234872 probe sets and 27478 metaprobe sets are defined in the full dataset. Differential expression of summarized gene level expression was calculated using the *f* test statistic followed by a false discovery rate multiple testing correction. Microarray data were deposited in Gene Expression Omnibus under accession number GSE105426.

Inclusion of CAKUT patients

Participants included 560 patients for DNA analysis derived from 3 cohorts: (i) A total of 461 patients derived from the Dutch AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children) data- and biobank project, which comprises DNA as well as clinical and questionnaire data on lifestyle and environmental factors from patients diagnosed with congenital malformations or childhood cancers and their parents.[46] Patients were diagnosed with CAKUT at the Radboud University Medical Center, Amalia Children's Hospital, Nijmegen, The Netherlands. (ii) Eighty-three patients were derived from a duplex collecting system/vesicoureteral reflux patient cohort that was described previously,[47] and (iii) 16 children with CAKUT were selected for mutational analysis in a multicenter approach. The inclusion criteria comprised renal hypodysplasia, double kidneys, and/or anomalies of the ureter with renal hypodysplasia defined by the presence of small kidneys (lower than the third percentile), and/or maldeveloped renal tissue on renal ultrasound. Sonographic criteria of maldeveloped renal tissue included a lack of corticomedullary differentiation and optional proof of renal cysts. Clinical information from the family members was collected wherever possible.

The study was approved by the ethics committees in all participating centers, and informed assent and/or consent for genetic screening was obtained from patients and/or parents, as appropriate. Peripheral blood samples were obtained, and DNA was isolated from lymphocytes using standard procedures. The patients and their diagnoses are summarized in Supplementary Table S1.

Mutation analysis of the human *SOX11* gene in CAKUT patients

The coding region and the intron-exon boundaries of the human *SOX11* gene (NM_003108.3) were analyzed by Sanger sequencing. Primers applied and PCR settings are available on request. In-house and the online available databases dbSNP, Ensembl genome browser, 1000 Genomes Project, and ExAC Browser were used to determine whether *SOX11* variants were detected previously. Evaluation of a functional effect of the variants was assessed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and mutation taster (http://www.mutationtaster.org).

Additional methods are provided in the Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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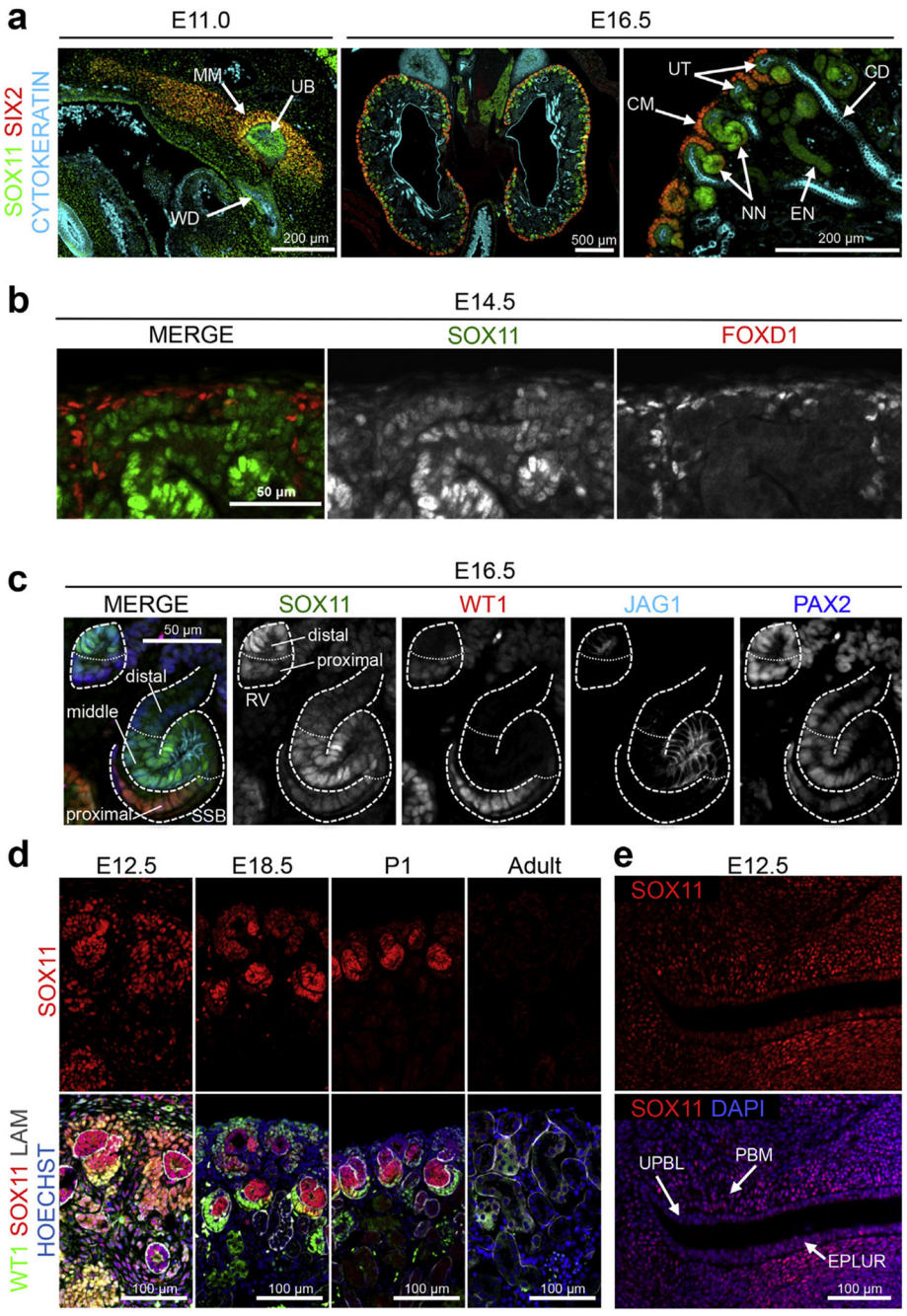

Figure 1 |. Dynamic expression of SOX11 during kidney development.

(**a**) Immunofluorescence analysis of wild-type embryos shows SOX11 expression in the metanephric mesenchyme (SIX2+) and Wolffian duct/ureteric bud (CYTOKERATIN+) at E11.0 (left panel). At embryonic day (E) 16.5, SOX11 expression is maintained at low levels in the Wolffian duct derivatives (ureteric tips and collecting ducts) and at high levels in the metanephric mesenchyme derivatives (cap mesenchyme and nascent nephrons) (middle and right panels). (**b**) High-power view showing that SOX11 is absent from FOXD1-positive stromal cells. (**c**) High magnification of a renal vesicle and S-shaped body of a wild-type

E16.5 embryo showing SOX11 expression in the proximal (WT1+), middle (JAG1+), and distal (PAX2+ JAG1− WT1−) segments. Note the higher SOX11 expression in the distal renal vesicle and middle SSB segments. (**d**) Immunostaining of SOX11 at different stages of kidney development (E12.5, E18.5, postnatal day (P)1, and adult). Simultaneous staining with WT1 and laminin has been used to visualize the different renal structures. (**e**) SOX11 is expressed in the urethra of E12.5 embryos. CD, collecting duct; CM, cap mesenchyme; DAPI, 4′,6-diamidino-2-phenylindole; EN, elongating nephron; EPLUR, epithelium of pelvic urethra; MM, metanephric mesenchyme; NN, nascent nephron; PBM, primitive bladder mesenchyme; RV, renal vesicle; SSB, S-shaped body; UB, ureteric bud; UPBL, urothelium of primitive bladder; UT, ureteric tip; WD, wolffian duct. Bars = 200 and 500 μm (**a**), 50 μm (**b,c**), 100 μm (**d,e**). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

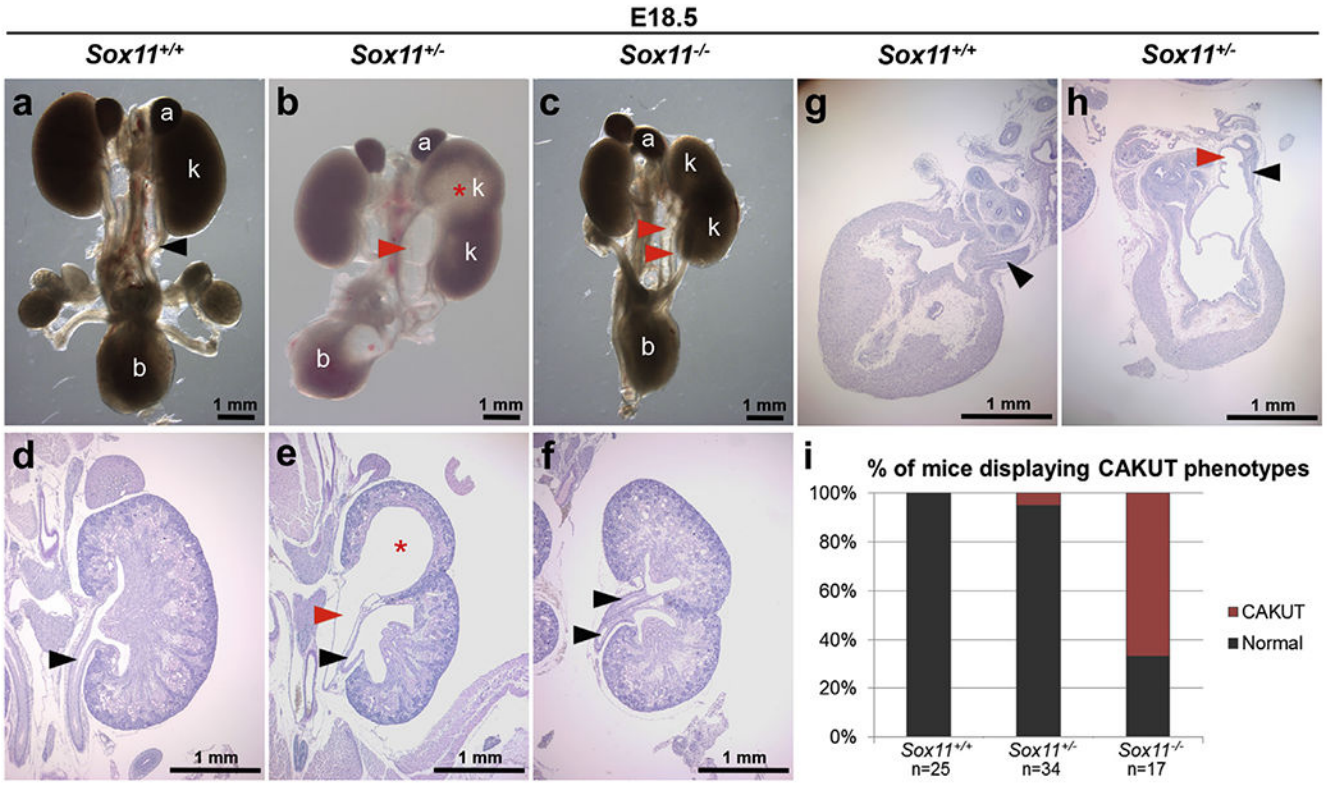

Figure 2 |. Kidney and ureter abnormalities in *Sox11* knockout mice.

Urinary tracts of embryonic day (E) 18.5 mice were processed as whole-mount (**a–c**) or with periodic acid–Schiff staining of tissue sections (**d–h**). Control $Sox11^{+/+}$ embryos (**a,d**) showing normal kidneys (k), adrenal glands (a), ureters (black arrowhead), and bladder (b). $Sox11^{+/-}$ (**b,e,h**) and $Sox11^{-/-}$ (**c,f**) embryos showing duplex kidneys, hydronephrosis (*), and hydroureter (red arrowheads). (**i**) Frequency of congenital abnormalities of the kidney and urinary tract (CAKUT) phenotype in $Sox11^{+/+}$, $Sox11^{+/-}$, and $Sox11^{-/-}$ embryos at postnatal day (P)0. Bar = 1 mm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

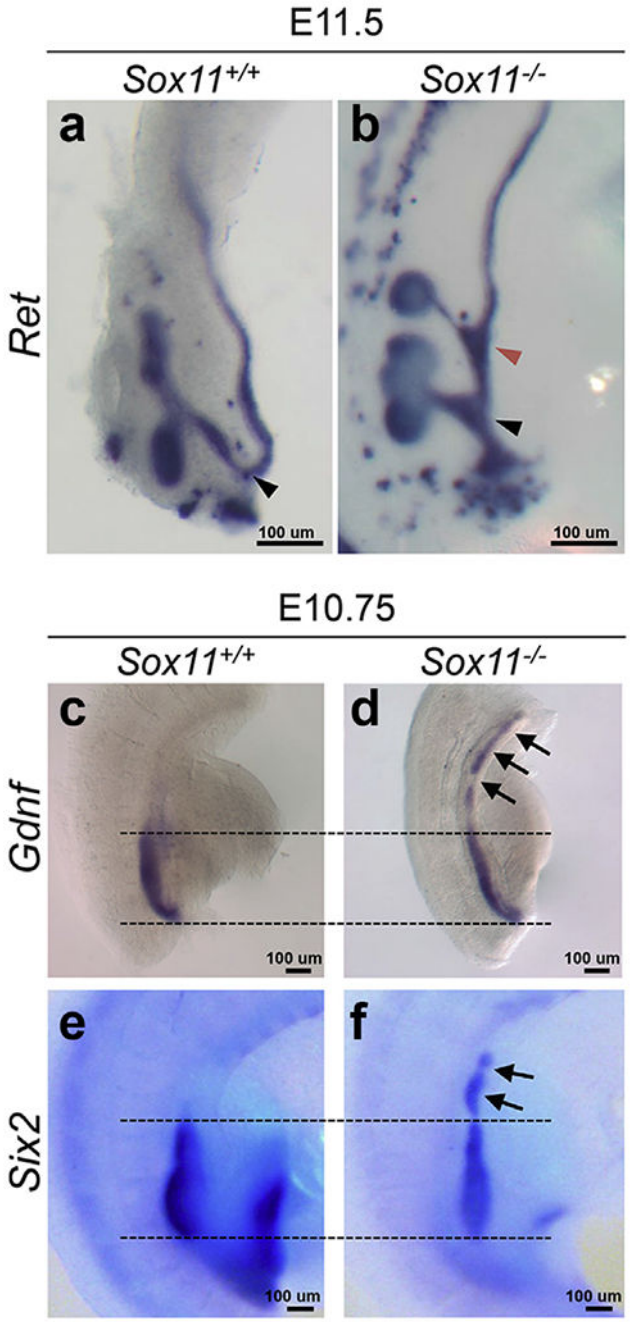

Figure 3 |. Loss of *Sox11* results in supernumerary ureteric buds and rostral extension of the *Gdnf* expression domain.

(**a,b**) Dissected urogenital tracts of embryonic day (E)11.5 mutant embryos were processed for whole-mount *in situ* hybridization using a *Ret* probe. A single ureteric bud (black arrowhead) has emerged and branched in the $Sox11^{+/+}$ embryo, whereas 2 ureteric buds (black and red arrowheads) had formed in the absence of *Sox11*. (**c–f**) Whole-mount *in situ* hybridization on urogenital tracts of E10.75 embryos using *Gdnf* and *Six2* probes shows a rostral extension of the *Gdnf* and *Six2* expression domain in $Sox11^{-/-}$ (black arrows, right

panel) compared with *Sox11*$^{+/+}$ (left panel). Bar = 100 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

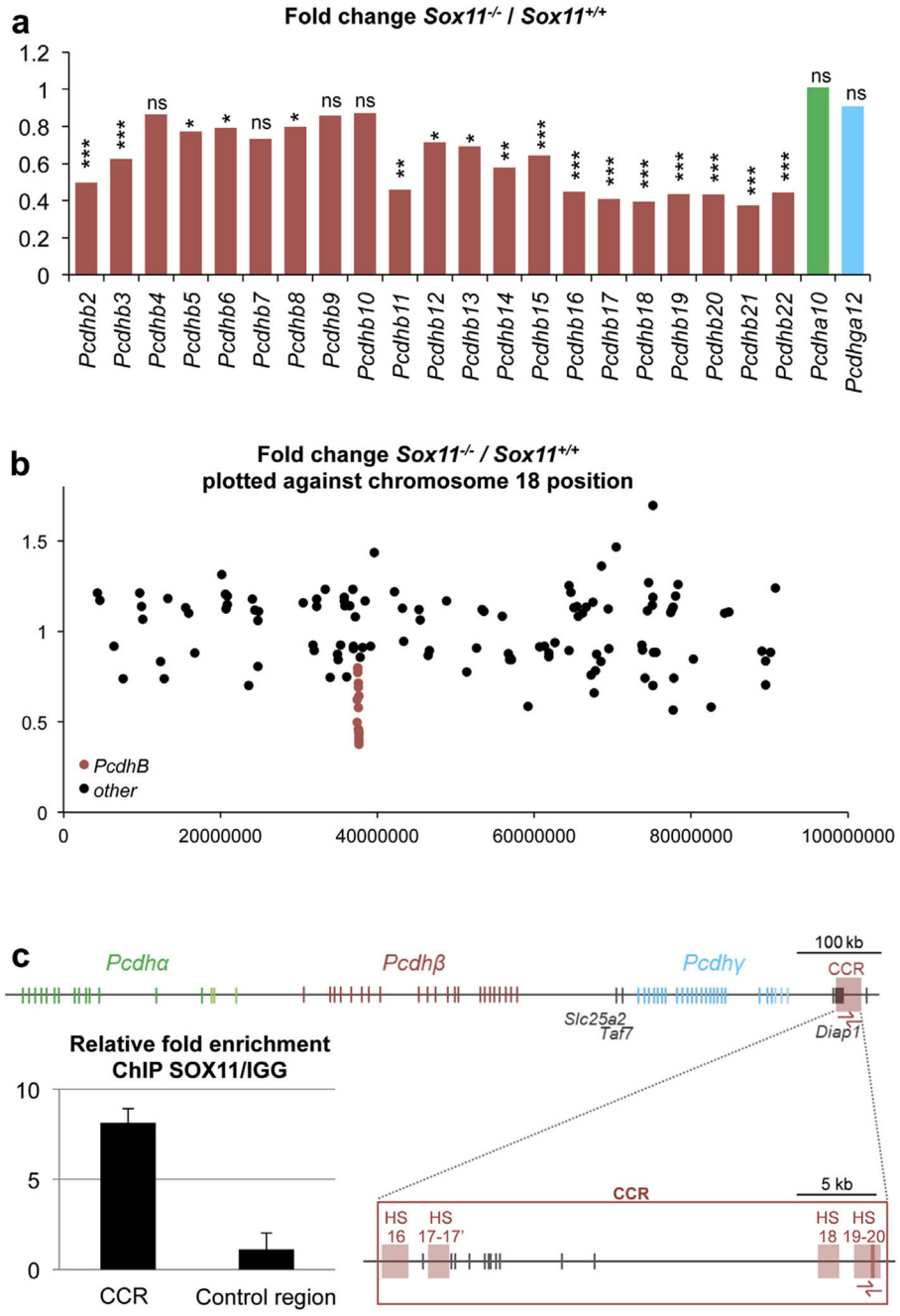

Figure 4 |. Microarray and ChIP analysis identifies the *protocadherin-β* cluster as a direct target of SOX11.

(**a**) Relative fold change in expression ($Sox11^{-/-}/Sox11^{+/+}$) of *protocadherin* genes obtained by microarray (*$P < 0.5$, **$P < 0.01$, ***$P < 0.001$). (**b**) Relative fold change expression ($Sox11^{-/-}/Sox11^{+/+}$) of chromosome 18 genes obtained by microarray ($P < 0.05$), plotted against their chromosomal location. (**c**) Schematic representation of the *Pcdh*-α (green), -β (red), and -γ (blue) loci. The *Pcdhβ*-cluster control region (CCR) is represented as a red box, containing 4 DNaseI hypersensitivity sites (HS16, 17-17′, 18, and 19-20). Red arrows

represent the primers used for ChIP analysis. ChIP-quantitative polymerase chain reaction analysis ($N = 2$) reveals an 8-fold enrichment of HS19-20 in SOX11-immunoprecipitated chromatin versus IgG-immunoprecipitated chromatin. No difference was seen on a control locus. ChIP, chromatin immunoprecipitation; HS, hypersensitivity site; ns, nonsignificant; *Pcdh*, protocadherin.

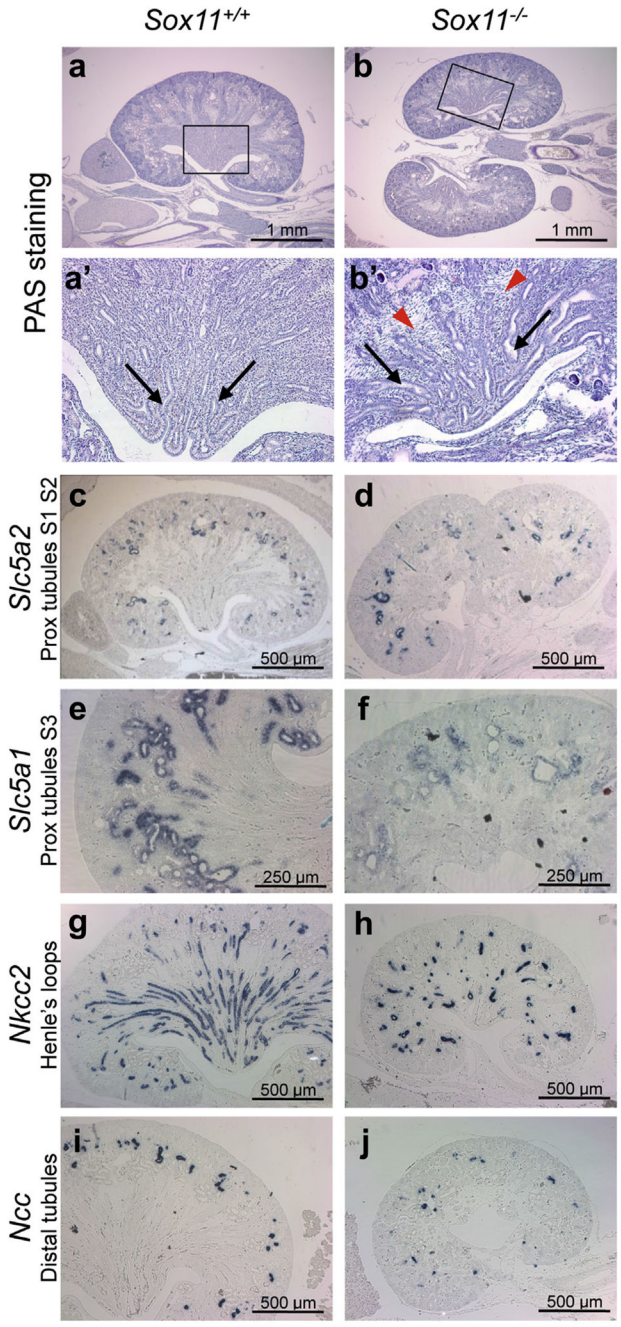

Figure 5 |. Loss of *Sox11* causes changes in renal architecture.

(**a,b**) Periodic acid–Schiff (PAS) staining of postnatal day (P)0 kidneys from wild-type and *Sox11*$^{-/-}$ animals. Closer views of the medullary zone (**a',b'**) show a reduction of epithelial components and an increase of stromal tissue in *Sox11* mutant embryos compared with controls. Black arrows point to the collecting ducts and red arrowheads to stromal cells. (**c–j**) *In situ* hybridization analysis of nephron segments in embryonic day 18.5 *Sox11*$^{+/+}$ (left panel) and *Sox11*$^{-/-}$ (right panel) embryos. *Slc5a2* (solute carrier family 5, member 2), *Slc5a1* (solute carrier family 5, member 1), *Nkcc2* (solute carrier family 12, member 1), and

Ncc (solute carrier family 12, member 3) riboprobes were used to visualize segments 1/2 of proximal (prox) tubules, segments S3 of proximal tubules, Henle's loops, and distal tubules, respectively. Bars = 1 mm (**a,b**), 250 μm (**e,f**), and 500 μm (**c,d,g–j**). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

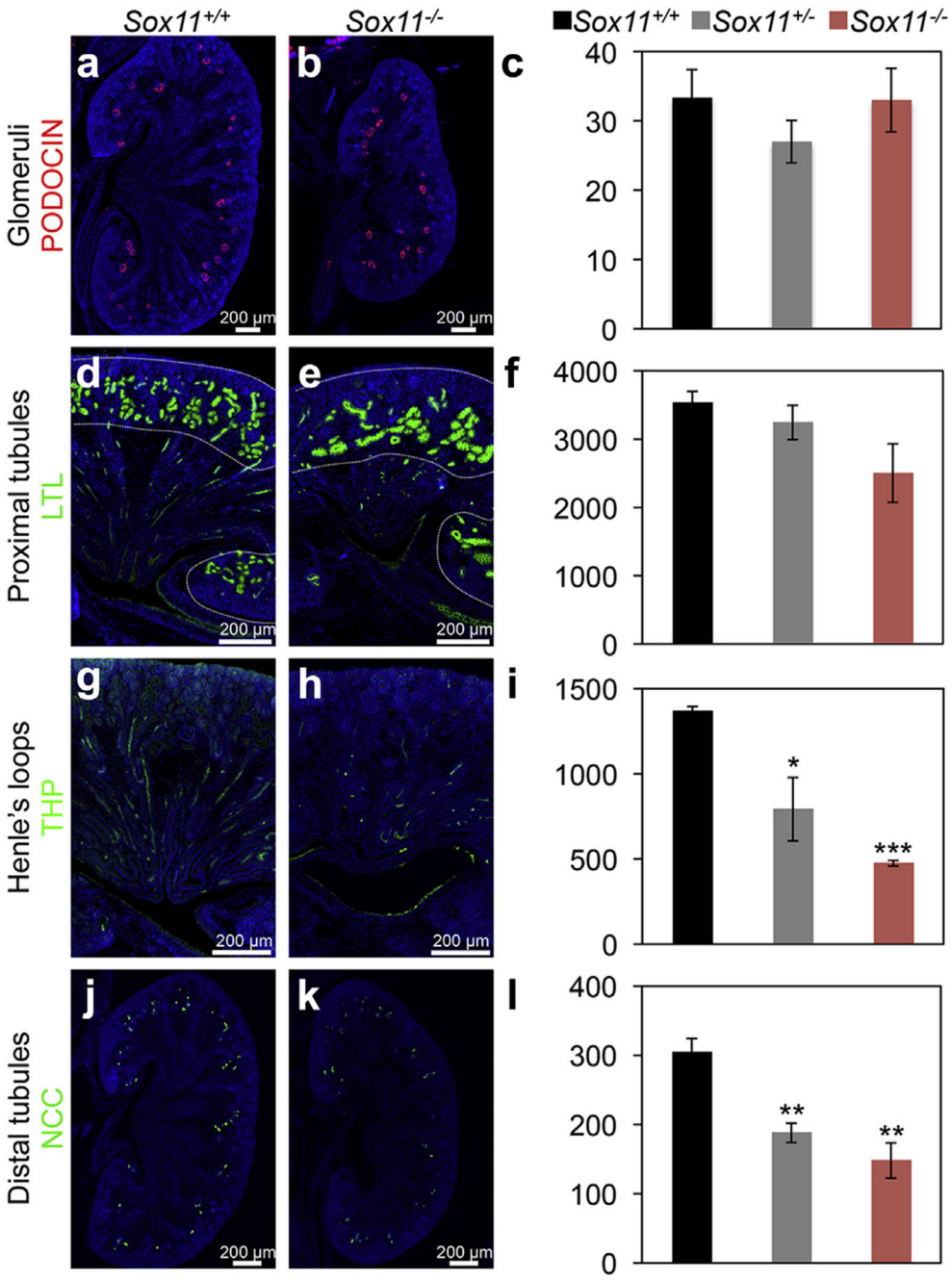

Figure 6 |. Nephron segment defects in *Sox11* mutant mice.

Immunofluorescence analysis of nephron segments in embryonic day (E) 17.5 *Sox11*$^{+/+}$ (left panel) and *Sox11*$^{-/-}$ (right panel) embryos. Podocin/NPHS2 (**a**,**b**), lotus tetragonolobus lectin (LTL) (**d**,**e**), Tamm-Horsfall protein (THP) (**g**,**h**), and NaCl cotransporter (NCC) (**j**,**k**) were used to visualize glomeruli, proximal tubules, Henle's loops and distal tubules, respectively. Dotted lines in **d** and **e** highlight the renal cortex containing the S1 and S2 segment of proximal tubules. (**c**,**f**,**i**,**l**) Quantification of glomeruli and cells in proximal (LTL+), Henle's loop (THP+) and distal (NCC+) tubules in *Sox11*$^{+/+}$, *Sox11*$^{+/-}$, and

Sox11$^{-/-}$ E17.5 embryos. Cells number is presented as the average with SEM of data obtained from 3 embryos of each genotype (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Bars = 200 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

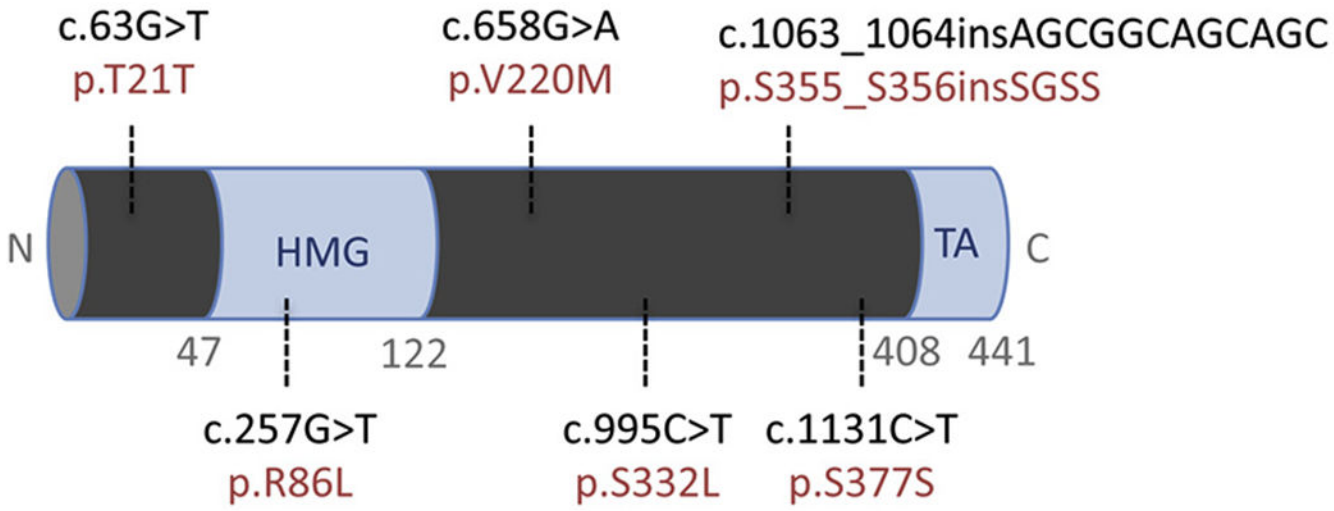

Figure 7 |. Coding sequence variants of *SOX11* found in CAKUT patients.

Linear representation of the human SOX11 protein showing the location of the variants identified in the CAKUT cohort. The coding sequence variations (black) and the corresponding amino acid changes (red) are indicated. Functional domains previously reported are displayed in blue: high-mobility group (HMG) DNA-binding domain (HMG aa.47-122) and transactivation domain (TA; TA aa.408-441). Amino acid positions are displayed in gray. C, C terminus; CAKUT, congenital abnormalities of the kidney and urinary tract; N, N terminus.

Table 1 |

Sox11 is required to ensure proper kidney development

				Duplex kidney				Hypoplasia	
Genotype	***N***	**Normal (%)**	**CAKUT (%)**	**Unilateral**	**Bilateral**	**Hydroureter**	**Hydronephrosis**	**Unilateral**	**Bilateral**
Sox11$^{+/+}$	26	26 (100)	0 (0)						
So11$^{+/-}$	39	37 (94.9)	2 (5.1)	2		2	2		
Sox1$^{-/-}$	21	7 (33.3)	14 (66.7)	7	5	2	1	2	2

CAKUT, congenital abnormalities of the kidney and urinary tract.

Frequency and detailed kidney phenotypes of *Sox11* knockout and *Sox11*$^{-/+}$ mutant mice. Note that hydroureter and hydronephrosis defects were always found in association with duplex kidney.

Table 2 |

Coding sequence variants of *SOX11* found in CAKUT patients

Patient	**1**	**2**	**3**	**4**	**5**		**6**
Sex	Male	Male	Female	Male	Male		Male
Country	The Netherlands	The Netherlands	The Netherlands	Germany	Germany		The Netherlands
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian		Caucasian
Phenotype	Posterior urethral valves	Multicystic /kidney dysplasia (left)	**Duplex collecting system (right)** **Ureteropelvic junction obstruction (right)** Vesicoureteral reflux (right)	Posterior urethral valves Multicystic kidney dysplasia (bilateral) Hepatic fibrosis Retinal degeneration Skeletal abnormalities	Posterior urethral valves **Kidney hypoplasia (bilateral)**		**Ureteropelvic junction obstruction (left)** **Maldescended testis (right)** Urethral meatal stenosis Hearing loss Glaucoma (bilateral)
Position on chromosome 2 (GRCh38.p2)	5692784	5692978	5693716	5693784	5693784	5693379	5693852
Coding sequence change (NM_003108.3)	c.63G>T	c.257G>T	c.995C>T	c.1063insAGCGGCAGCAGC	c.1063insAGCGGCAGCAGC	**c.658G>A**	c.1131C>T
Type of mutation	Silent	Missense	Missense	Insertion	Insertion	Missense	Silent
Amino acid change (NP_003099.1)	p.T21T	p.R86L	p.S332L	p.355insSGSS	p.355insSGSS	p.V220M	p.S377S
Variant dbSNP ID	rs139885563	rs202211730	Novel	Novel	Novel	rs758958162	rs762687158 (not validated)
Allele frequency							
ExAC (non-Finnish Europeans)	0.001	—	—	—	—	0.0003	0.00002
Our CAKUT cohort	0.0009	0.0009	0.0009	0.002	0.002	0.0009	0.0009
PolyPhen-2 prediction	—	Probably damaging	Probably damaging	—	—	Probably damaging	—
Score	—	0.996	0.991	—	—	0.996	—

Patient	**1**		**2**	**3**		**4**		**5**		**6**	
Sensitivity	—		0.55	0.71		—		—	0.55	—	
Specificity	—		0.98	0.97		—		—	0.98	—	
Mutation Taster											
Prediction	—		Disease causing	Disease causing		—		—	Disease causing	—	
Prob	—		0.999	0.999				—	0.996	—	
SIFT											
Prediction	—		Tolerated	Tolerated				—	Tolerated	—	
score	—		0.46	0.14					0.11	—	
RegRna prediction											
Motif type	Intron splicing enhancer	Functional RNA sequences	Functional RNA sequences	Transcriptional regulatory motif	Transcriptional regulatory motif	Human splicing sites	Human splicing sites	Transcriptional regulatory motif	Intron splicing enhancer	Human splicing sites	Intron splicing enhancer (ISE)
Motif name	gh-1 intron 3	FR084090/ group I intron	FR012169/Putative conserved_noncoding_region_(EvoFold)	HIC1	BEN	Donor	Acceptor	BEN	Amyloid precursor protein, exon 8	Donor	ighg2 cgamma2 (IgG heavy chain subclass g2 - cgamma2 gene) - intron 1
Change in variant versus wild type	Suppressed	Suppressed	Additional	Additional	Additional	Suppressed	Additional	Additional	Additional	Additional	Additional

SOX11 variants identified in CAKUT cohort, their associated phenotypes, frequency in our CAKUT cohort and the ExAC database, and *in silico* mutation prediction results. Patient phenotypes that were also displayed by the *Sox11* knockout are shown in bold.