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Supplemental Data

Rare Variants in *BNC2* Are Implicated in Autosomal-Dominant Congenital Lower Urinary-Tract Obstruction

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Supplemental note: Case report of Family 1

The male index individual (IV-2) was born spontaneously after preterm labor at 32+1 weeks of gestation to a 39-year-old gravida 3, para 0 mother, following a pregnancy complicated by early diagnosis of severe LUTO at 13 weeks of gestation. In order to prevent intrauterine renal insufficiency, vesicoamniotic shunting was performed directly after prenatal diagnosis at 13 weeks of gestation. Postnatal micturition cystourethrography at three weeks of life showed severe urethral obstruction suggesting PUV (Figure 1 **D**). Later surgical reconstruction with creation of a tubularized neourethra at nine months of age revealed the final diagnosis of urethral stenosis. The mother of the index individual (III-4) had a history of recurrent urinary tract infections starting at 12 years of age. In addition, she had early onset of increased urinary daytime frequency and nocturia with up to eight times per night. Urological workup at the age of 16 years including urodynamic testing revealed an underlying urethral stenosis. She rejected therapeutic urethral bouginages leaving her with a persistent increased urinary daytime frequency and nocturia at the time of assessment. Her first two pregnancies ended at nine and ten weeks of gestation respectively with spontaneous abortion of unknown cause (Figure 1 **C**). At the time of assessment, the maternal grandmother (II-4) of the index individual was 65 years. She also had an increased urinary daytime frequency and nocturia of up to five times per night. At 47 years she started to have urinary incontinence. Urological workup at that age revealed slight descensus of her bladder and distal urethral stenosis (meatal stenosis). The sister of the maternal grandmother (II-2) was 72 years of age at the time of assessment. She had one spontaneous abortion at nine weeks of gestation. She denied having voiding dysfunction but did not consent to any urological or genetic

assessment. In accordance with the previously described well-noted high within-family phenotypic variability, we considered her to be healthy. In contrast, her 43 year old daughter (III-2), had a history of early onset increased urinary daytime frequency and nocturia with urinary tract infections in her youth and was finally diagnosed with high grade urethral stenosis at 42 years of age which was surgically corrected at that time. Of note, the maternal great-grandmother (I-2) of the index individual (IV-2), had one spontaneous abortion at eight weeks of gestation and two living daughters (II-2 and II-4).

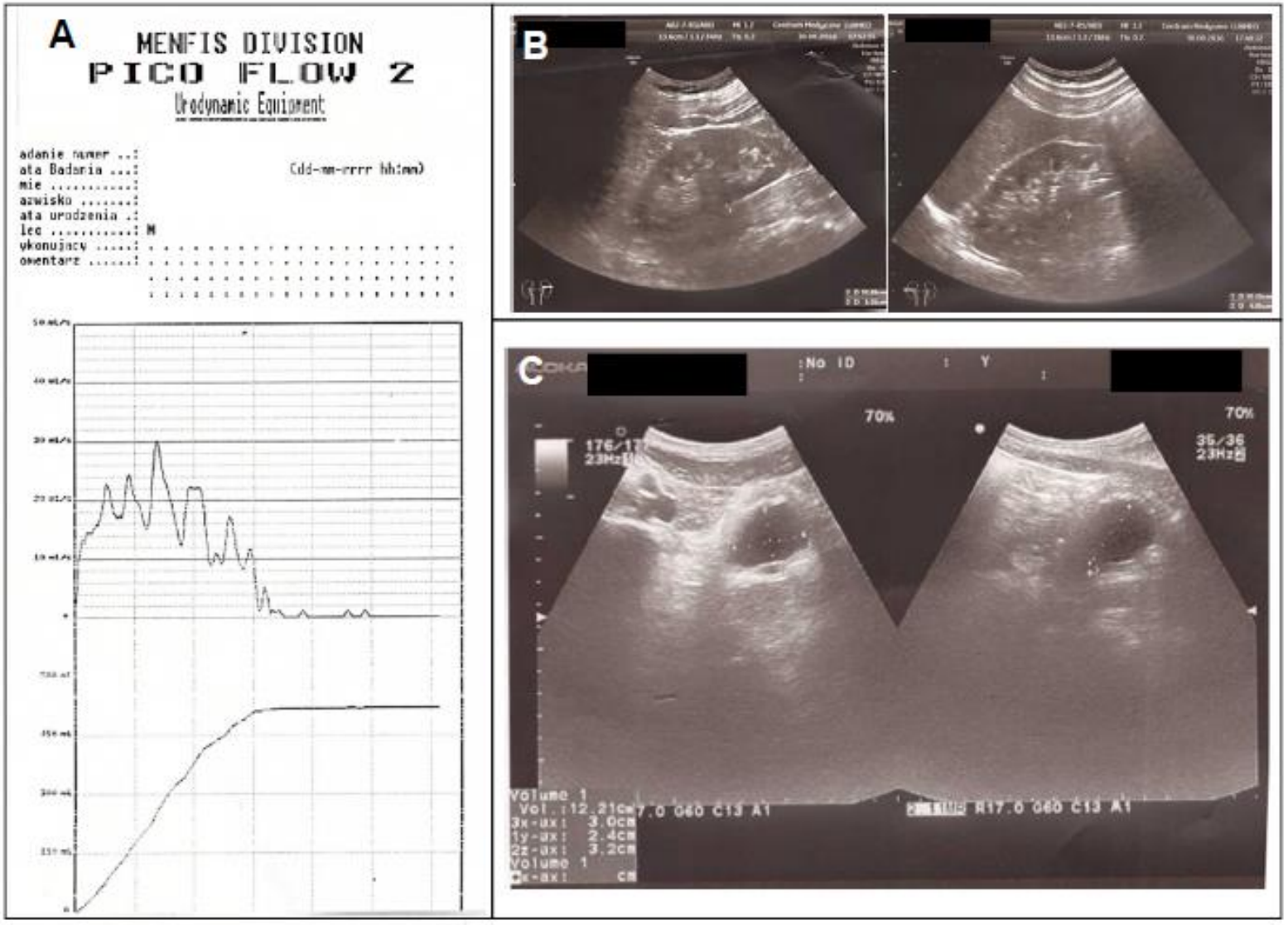

A
MENFIS DIVISION
PICO FLOW 2
Urodynamic Equipment
adanie numer ..:
ata Badania ...:
(dd-mm-rrrr hh:mm)
mie .....:
azwisko .....:
ata urodzenia .:
lec .....: M
ykonujacy .....:
omentarz .....:
B
C
:No ID
:
Y
70%
70%
35/36
23Hz
Volume 1
Vol.:12.21cm
3x-ax: 3.0cm
1y-ax: 2.4cm
2z-ax: 3.2cm
Volume 1
R17.0 060 C13 A1

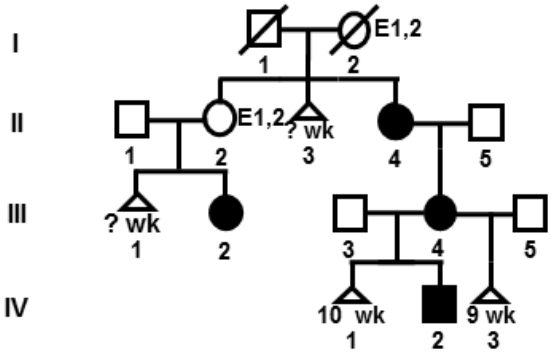
Figure S1: Pathological Uroflowmetry of Individual II-1 in Family 2

(**A**): Uroflowmetry showing dysfunctional voiding, voided urine volume = 520 ml.

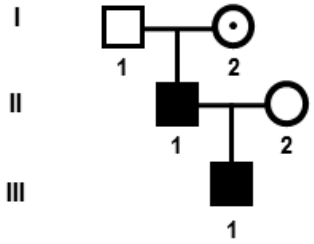
(**B**): Normal renal ultrasound of right and left kidney.

(**C**): Ultrasound of bladder showing 12 ml of residual urine after uroflowmetry.

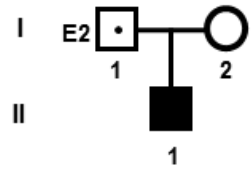
A **Family 1** (c.2557C>T, p.R853*, ENST00000418777)



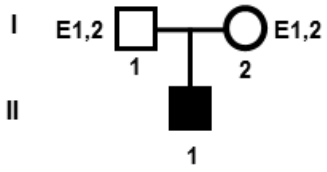
B **Family 2** (c.2663A>G, p.H888R, ENST00000380672)



C **Family 3** (c.473C>T, p.T158I, ENST00000380672)



D **Family 4** (c.1036G>C, p.E346Q, ENST00000380672)



E **Family 5** (c.13G>A, p.V51I, ENST00000545497)

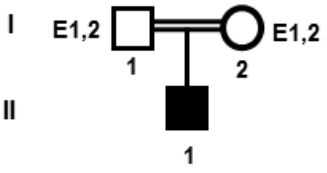


Figure S2: Pedigrees of Identified Families with LUTO Phenotype with Rare Variants in *BNC2*

(A): Pedigree with multiple affected individuals in the index Family 1. Affection of Individuals varies in severity: II-4: meatal stenosis, pollakisuria and nycturia; III-2: urethral stenosis, surgically corrected, frequent urinary tract infections (UTI), pollakisuria and nycturia; III-4: urethral stenosis, frequent UTIs in youth, pollakisuria and nycturia; IV-2: prenatal diagnosis in 13th week of gestation with megacystis, urethral stenosis, surgically corrected/reconstructed. Individuals II-2 and I-2 are more precisely highlighted with the symbol E1,2 for unavailable genetic and clinical evaluation, representing potential carriers of the *BNC2* variant but did not participate in our study. Triangles denote miscarriages and are annotated with respective weeks of gestation. E1 = unavailable genetic evaluation; E2 = unavailable clinical evaluation; Wk = week of gestation

(B): Pedigree with multiple affected individuals of Family 2. Carriers of the identified *BNC2* variant present with varying severity: I-2 no history of UTI or high voiding frequency, normal renal ultrasound; II-1: pollakisuria, nycturia and pathological MCUG; II-1: posterior urethral valve. I-2 is highlighted with a dot, representing a healthy carrier of the identified *BNC2* variant.

(C): Pedigree with one affected individual of Family 3. II-1 with the identified *BNC2* variant presents with posterior urethral valves, vesico ureteral reflux grade 1-2.5, bladder diverticles. I-1 is a carrier of the same variant, but was unavailable for clinical evaluation (E2).

(D): Pedigree with one affected individual of Family 4. II-1 with the identified *BNC2* variant presents with posterior urethral valves, right hydronephrosis, left vesico ureteral reflux

grade 1, incontinence until 6 years of age. The parents I-1 and I-2 were unavailable for genetic and clinical evaluation (E1,2).

(E): Pedigree with one affected individual of Family 5. II-1 from a consanguineous descent with the identified *BNC2* variant presents with posterior urethral valves, vesico ureteral reflux, end stage renal disease. The parents I-1 and I-2 were unavailable for genetic and clinical evaluation (E1,2).

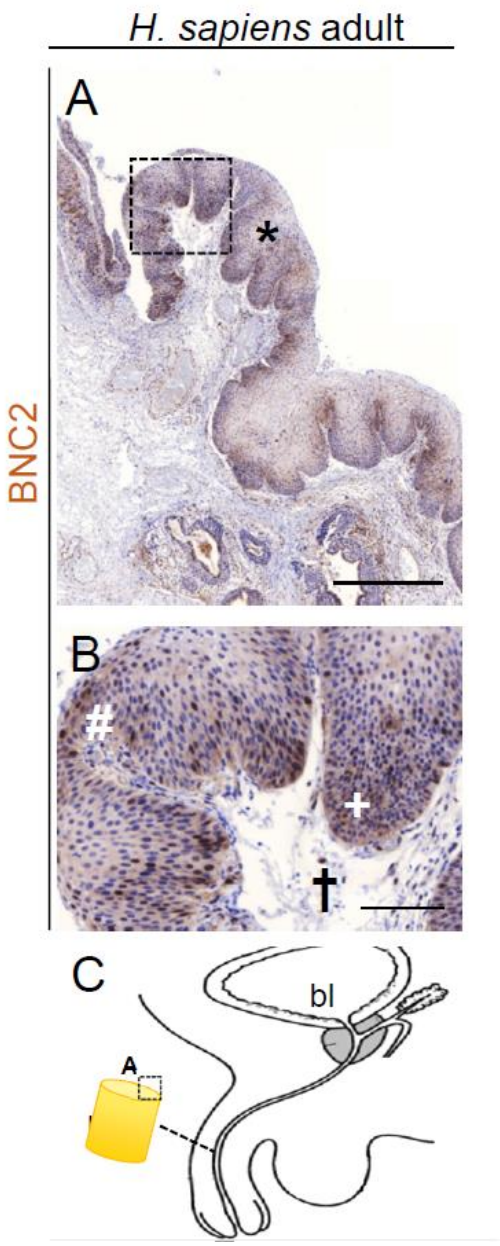

H. sapiens adult
BNC2
A
*
B
#
+
†
C
bl
A

Figure S3: Expression of Human BNC2 in Adult Male Urethra

(**A-B**): Immunohistochemistry staining for BNC2 on a transverse section of adult human distal urethra (positive stained tissue is marked with an asterisk *). (B) Magnification (square in A) of the basal layer of the squamous mucosa epithelium shows a weak cytoplasmic positive reaction with the antibody against BNC2 (+). Furthermore, a diffuse nuclear staining in all layers of the epithelium can be observed (#). In the stroma are some plasma cells with a positive staining (†).

(**C**): Schematic overview of the human male urogenital organ depicting the location of tissue (dashed square) shown in A. bl = bladder, peur =penile urethra.

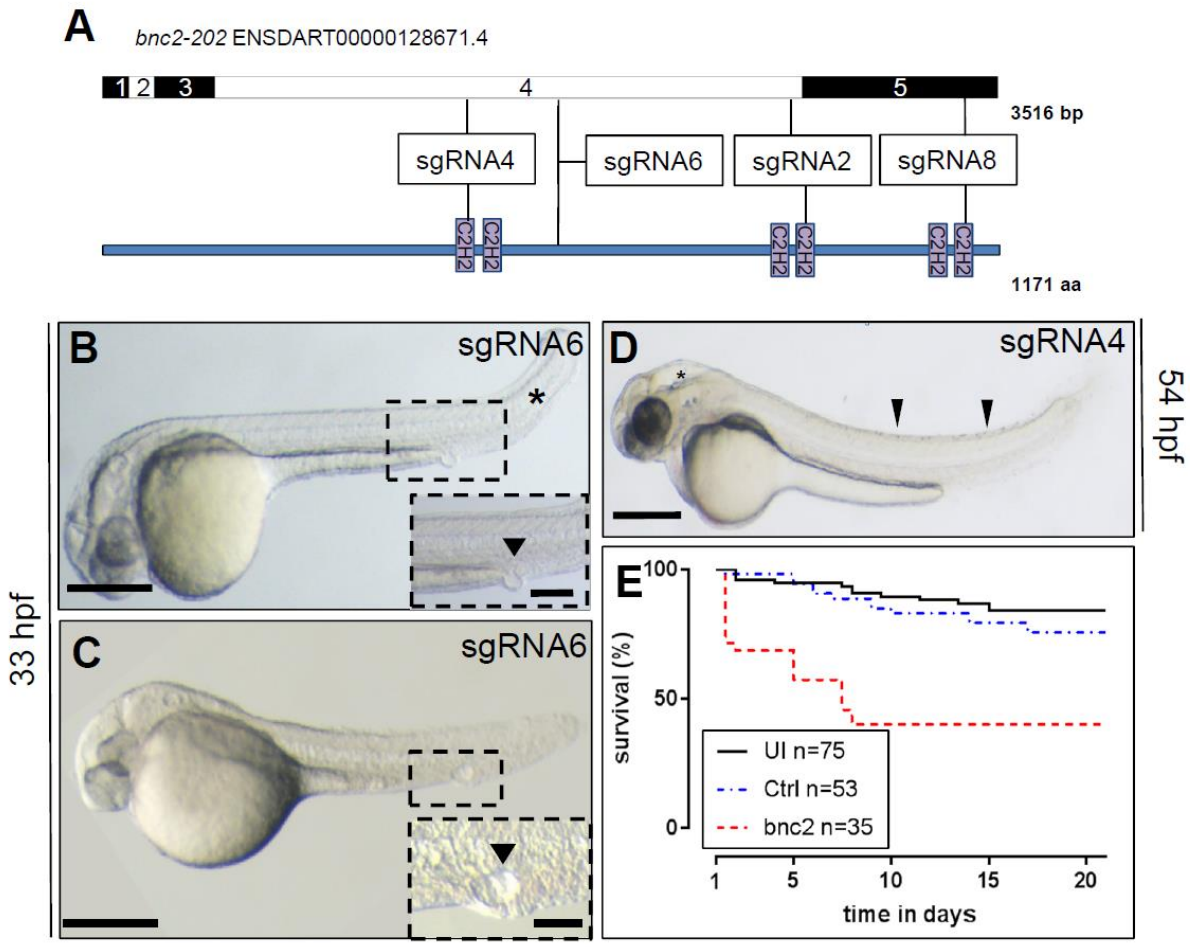

A
bnc2-202 ENSDART00000128671.4
1 2 3 4 5
3516 bp
sgRNA4
sgRNA6
sgRNA2
sgRNA8
C2H2
1171 aa
B
sgRNA6
C
sgRNA6
D
sgRNA4
54 hpf
33 hpf
E
survival (%)
100
50
0
UI n=75
Ctrl n=53
bnc2 n=35
1
5
10
15
20
time in days

Figure S4: Transient Bnc2 CRISPR/Cas9 Knockdown in Zebrafish Larvae Reproduces the Morpholino Phenotype

(**A**): Schematic showing the genomic positions of the different guide-RNAs used for the transient Bnc2 CRISPR/Cas9 targeting experiments.

(**B-C**): Single guide RNA (sgRNA) injections of guide 6 targeting *bnc2* with Cas9 reproduces the distal 'vesicle' as an outlet obstruction of the pronephric ducts in 5 % of injected embryos at 33 hpf (see inlays - black arrowheads). Kinked tails were observed in 1 % of the zfl (black asterisk).

(**D**): Follow-up of sgRNA injected zfl targeting *bnc2* for sgRNA 4 shows hydrocephalus (black asterisk) and light body curvature (black arrowheads) at 54 hpf.

(**E**): Kaplan-Meier plots for survival of acute multi sgRNA (mgRNA) approach for guides 2, 6 and 8 targeting *bnc2* with Cas9. Zfl were included in survival experiment after 24 hpf. Injected larvae show significantly ($p<0.0001$, Mantel-Cox test) reduced survival (median survival of 7 dpf) and 40 % survival at the end of the experiment.

Scale bars: 500 µm (B, C and D)

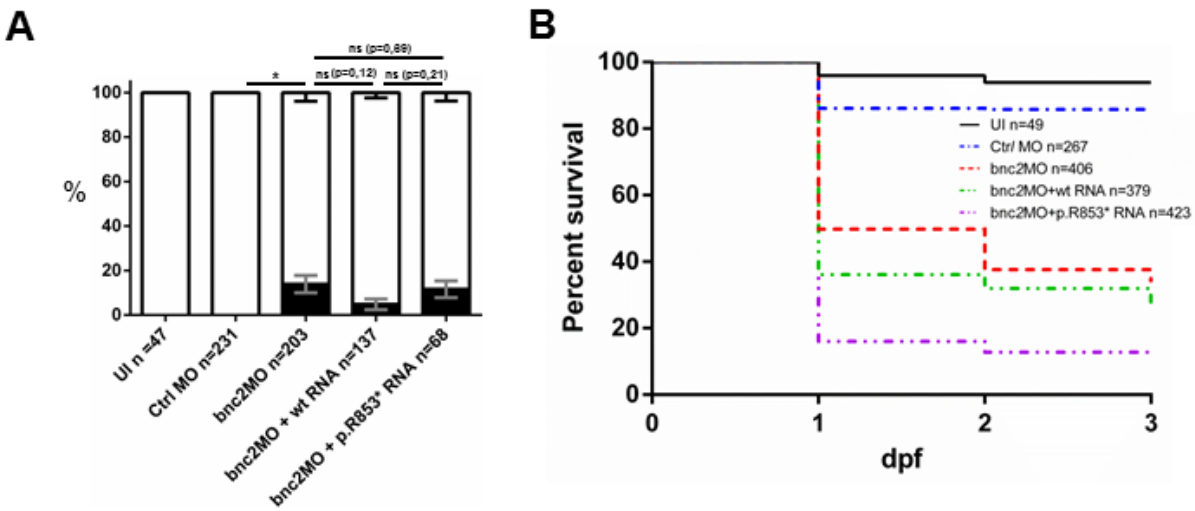

A
ns (p=0,89)
*
ns (p=0,12)
ns (p=0,21)
100
80
60
40
20
0
%
UI n =47
Ctrl MO n=231
bnc2MO n=203
bnc2MO + wt RNA n=137
bnc2MO + p.R853* RNA n=68
B
Percent survival
100
80
60
40
20
0
0
1
2
3
dpf
UI n=49
Ctrl MO n=267
bnc2MO n=406
bnc2MO+wt RNA n=379
bnc2MO+p.R853* RNA n=423

Figure S5: Co-injection of *bnc2* MO and Human Wildtype RNA in Zebrafish Larvae Results in a Reduced Formation of a Distal Pronephric Outlet Obstruction

(**A**): The graph shows 100 % of all at 1 dpf surviving zebrafish larvae (zfl) of the five different cohorts uninjected (UI), control Morpholino (Ctrl MO), *bnc*2 MO, *bnc2* MO + wildtype (wt) RNA (transcript ENST00000418777) and *bnc2* MO + mutated RNA bearing the p.R853* variant (for absolute numbers see Figure S5 A.) A distal 'vesicle' due to an outlet obstruction (phenotype) of the pronephric ducts can be seen in 14 % of *bnc2* MO injected zfl compared to 0 % of zfl with a 'vesicle' in both control groups ($p<0.05$, unpaired t-test). Zfl injected with *bnc2* MO + wt RNA develop a 'vesicle' in 5 %, injected with *bnc2* MO + p.R853* RNA in 11 %. * significance $p < 0.05$

(**B**): Quantification of death rates up to 3 dpf shows an increased mortality up to 66 % in *bnc2* MO injected zfl compared to UI (6 %) and Ctrl MO (14 %) injected zfl. Injected *bnc2* MO zfl show significantly ($p<0.0001$, Mantel-Cox test) reduced survival. Co-injection of *bnc2* MO + human wt RNA results in a mortality of 73 %. Aggravated mortality up to 88 % can be detected in the *bnc2* MO + p.R853* RNA injected zfl.

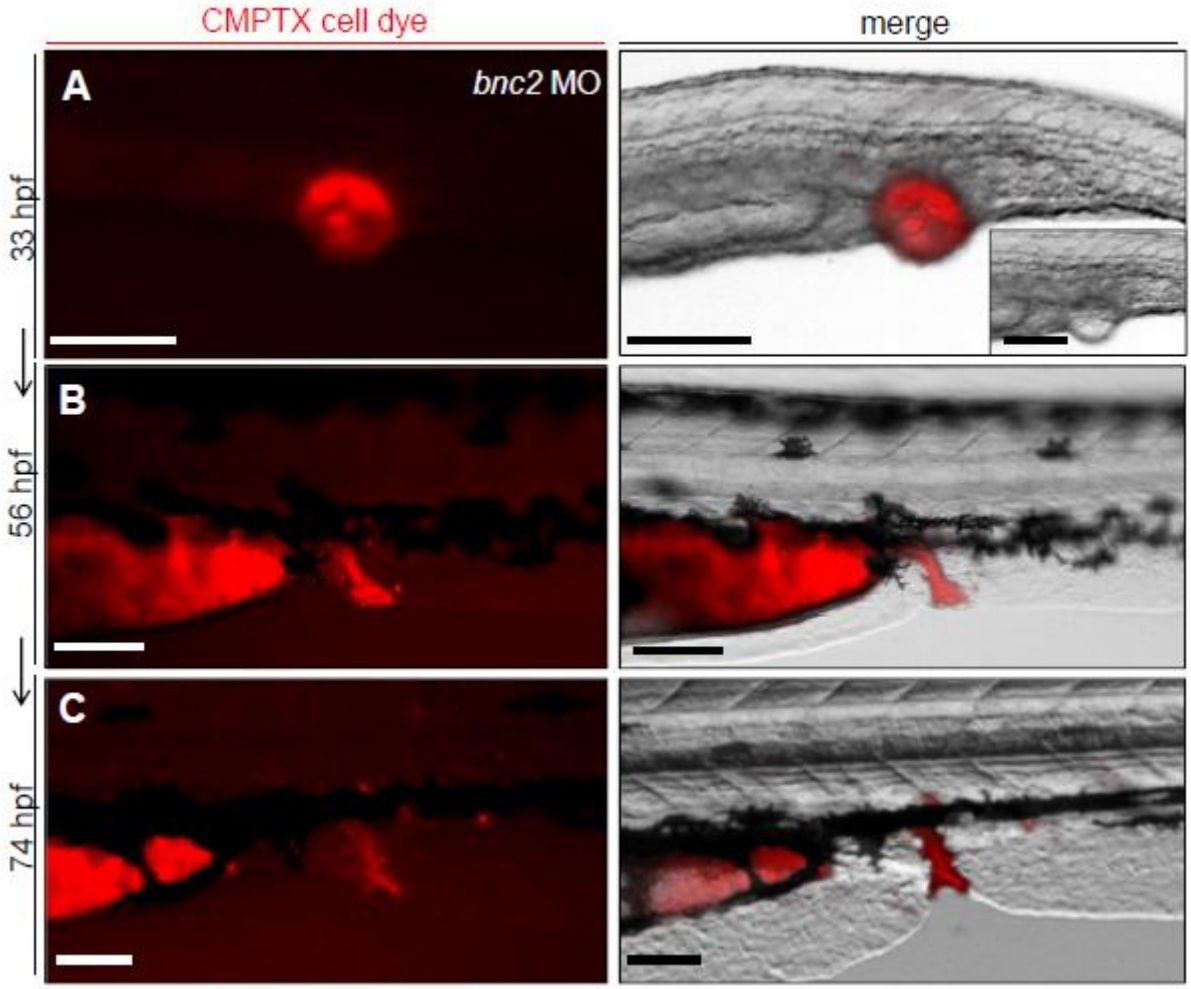

CMPTX cell dye
merge
A
bnc2 MO
B
C
33 hpf
56 hpf
74 hpf

Figure S6: Injection of Fluorescent Cell Dye Tracker CMTPX Indicates Surrounding Cells of Pronephric Outlet Obstruction to Later Form Parts of Distal Pronephric Ducts and the Cloaca

(**A-C**): Injection of fluorescent cell dye tracker CMPTX into the pronephric outlet obstruction ‘vesicle’ of a *bnc2* MO injected zebrafish embryo at 33 hpf and follow-up imaging of the same individual zebrafish demonstrates that cells forming the ‘vesicle’ become part of the distal end of the pronephric ducts and the cloaca later in development (56 and 74 hpf).

Scale bar: 100 μm (A, B and C)

Table S1: All Variants Segregating with Disease in Family 1 Identified by Exome Sequencing with a Frequency < 0.0001 According to gnomAD

	HGNC	RefSeq	Mutation cDNA	Mutation Protein	Allele frequency according to gnomAD
Loss of function variants					
ENST00000418777	*BNC2*	NM_001317939.1	c.2557C>T	p.Arg853*	0.000028
ENST00000295897	*ALB*	NM_000477.5	c.8G>A	p.Trp3*	*Novel*
Missense Variants					
ENST00000261405	*VWF*	NM_000552.3	c.7486C>A	p.Pro2496Thr	*Novel*
ENST00000397016	*CPNE6*	NM_006032.2	c.47C>T	p.Thr16Met	0.000009
ENST00000336395	*TESK1*	NM_006285.2	c.427T>G	p.Ser143Ala	*Novel*
ENST00000324001	*PRX*	NM_181882.2	c.1546C>T	p.Arg516Trp	0.000098
ENST00000372584	*HIVEP3*	NM_001127714.2	c.3611A>T	p.His1204Leu	0.000031
ENST00000311303	*ABCC12*	NM_033226.2	c.2957C>T	p.Pro986Leu	0.000008
ENST00000284061	*DGKE*	NM_003647.2	c.487G>A	p.Val163Ile	*Novel*
ENST00000295890	*COX18*	NM_173827.2	c.782G>A	p.Arg261His	0.00005
ENST00000378113	*C12orf42*	NM_198521.2	c.383G>C	p.Arg128Pro	*Novel*
ENST00000392839	*RIC8B*	NM_018157.2	c.1018G>C	p.Glu340Gln	0.000008
ENST00000278935	*CEP164*	NM_014956.4	c.4130C>T	p.Pro1377Leu	0.000083

Legend Table S2 (excel file):

[1] nucleotide, exon and amino acid numbering for *BNC2* is according to ENST00000380672.8; the cDNA position is according to the transcript with 5'UTR; IVS=intervening sequence (intron); [2] estimation of consensus splice site values (CVs) is according to Shapiro and Senapathy[6]; [3] ESS, exonic splice silencer; [4] ESE, exonic splice enhancer; threshold for: SRSF1, 1.956; SRSF1 (IgM-BRCA1), 1.867; SRSF2, 2.388: SRSF5, 2.67; SRSF6, 2.676

Supplemental Methods

Subject Ascertainment and Phenotypic Data of LUTO Re-sequencing Cohort

The study was conducted in adherence to the Declaration of Helsinki. The respective informed consent was obtained from the affected individuals or by proxies in the case of minors. The study was approved by the ethics committee of the medical faculty of the university of Bonn (No. 146/12) as well as the respective ethic committee of the collaborating centers in Boston, Manchester, Nijmegen (AGORA data and biobank). Pre- and postnatally diagnosed affected individuals were included in the cohort screened for mutations in *BNC2*. Prenatal inclusion criteria comprised the presence of a distended bladder with 'keyhole sign', and with vesicoureteral reflux including uni- or bilateral hydronephrosis of different degree. Postnatal diagnosis was made by the presence of a valve or stenosis at time of cystoscopy. Clinical data were obtained by direct sonographic or radiographic studies of all participants at the respective clinical centers or retrospective reviewing of the medical files from the respective treating physician.

For re-sequencing we used DNA of 697 individuals with LUTO. All of these 697 individuals with LUTO were of male gender. In 13 affected individuals with distended bladder pregnancy was aborted, exact origin of LUTO was not clarified. All other affected individuals presented postnatally with PUV, except for one individual who was described to have an anterior valve.

Variant Identification

Whole-exome sequencing was performed with genomic DNA extracted from peripheral blood, captured (Agilent SureSelect Human All Exon v5) and sequence data were

generated by a paired end 2 × 125 bp protocol on the Illumina HiSeq 2000. This resulted in a mean coverage of 80-fold, a 30x coverage of 91 %, and a 10x coverage of 98 % of target sequences. Scripts developed in-house at the Cologne Center for Genomics (CCG) were applied to detect protein changes, affected donor and acceptor splice sites, and overlaps with known variants. Acceptor and donor splice site mutations were analyzed with a Maximum Entropy model [1] and filtered for effect changes. In particular, we filtered for high-quality (minimal coverage >6; minimal quality >10) and heterozygous variants with a frequency below 0.0001 since we hypothesized an autosomal-dominant mode of inheritance. (dbSNP build 135, the 1000 Genomes database build 20110521, ExAc Browser Version 0.3.1, and the public Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, build ESP6500). We also filtered against an in-house database containing variants from 511 exomes from individuals with epilepsy to exclude pipeline related artefacts (MAF<0.004). In a second step all remaining variants were checked for frequency in gnomAD version 2.0.2. Visual inspection of read quality was performed.

Re-sequencing of *BNC2* in Individuals with LUTO

Analysis of the human *BNC2* gene comprising 14 transcripts (ENST00000380672.8, ENST00000545497.5, ENST00000418777.5, ENST00000380667.6, ENST00000411752.5, ENST00000380666.6, ENST00000603713.5, ENST00000471301.3, ENST00000486514.5, ENST00000617779.1, ENST00000468187.6, ENST00000613349.4, ENST00000603313.5, ENST00000484726.5) listed in ‘ensembl database’ (www.ensembl.org/_September 30th 2017) was performed using Sanger Sequencing. PCR-amplified DNA products (primer

sequences available upon request) were subjected to direct automated sequencing using a 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) according to the manufacturer's specifications, with PCR primers also served as sequencing primers.

Staining of Human Urethra

For our study, we have used the formalin-fixed paraffin embedded archive. Initially, tissues were fixed in the neutral buffered 4% formalin according to the institutional standards. Human embryonic material, collected with maternal consent and ethical approval (REC 08/H0906/21+5), was sourced from the MRC-Wellcome Trust Human Developmental Biology Resource (http://www.hdbr.org/).

Hematoxylin and Eosin (HE) Staining

All samples were stained by the hematoxylin and eosin (HE) method following the standard protocol. Sections of 3 µm thickness were obtained from the paraffin blocks. Subsequently, the sections were dewaxed in xylene, hydrated in solutions with decreasing concentrations of ethanol, stained, dehydrated in solutions with increasing concentrations of ethanol and mounted from xylene with pertex from Medite.

Immunohistochemistry Protocol

The tissue was cut (3 µm) and mounted on superfrost slides (Menzel Gläser, Brunswick). The immunohistochemical staining of the 3 µm cuts is performed on the fully integrated staining solution Benchmark Ultra from Roche/Ventana.

The slides were pretreated with CC1 (cell conditioning pH 8) (Roche Ultra-CCl, 950-224). Antibodies incubation time was 36 min at 37° C. We used mouse monoclonal Anti-BCN2 antibody, clone 2082C5a, Abcam 84845, dilution 1:50. The ultraView Universal DAB Detection Kit from Roche (760-500) was used. We used counterstaining with hematoxylin according to Mayer (3 min) then ascending alcohol series. The slides from xylene were mounted with pertex from Medite.

Immunohistochemistry Evaluation

The immunohistochemically staining was evaluated by two experienced pathologists. The staining intensity was evaluated using a 4-tier grading system (0: negative; 1: weakly positive; 2: moderately positive; 3: strongly positive) for membrane, nuclei and cytoplasm separately.

Mouse *In Situ* Hybridization and mRNA Sequencing

Presence of the different *Bnc2* mRNAs (*Bnc2-201* and *Bnc2-214*) in the urogenital region of the developing mouse embryo was verified by PCR. To this end, the urogenital region from three E12-5 mouse embryos was prepared and RNA extracted using the RNeasy Mini Kit (Qiagen) according to the standard protocol. 500ng of RNA was translated into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The two transcript variants (*Bnc2-201*, ENSMUST00000102820.8 and *Bnc2-214*, ENSMUST00000176612.7) were detected with specific primers: *Bnc2-201*: forward: AAGAGATGCACGTCTGCACG, reverse: GTGTAGACACAGAGGCACACA; *Bnc2-214*: forward: ACAGAAACAGAAATTTACGGATGGA, reverse:

CGGAAGCACACACTGGCTAT. The obtained PCR products were sequenced for verification.

In situ probe generated by addition a 5' T7 polymerase transcriptional initiation sequence (aTAATACGACTCACTATAGGGG) to the reverse primer (*Bnc2-214* above). The ~800bp long PCR product was directly used for the antisense probe generation using the T7 RNA polymerase and a nucleotide mix containing digoxigenin-11-UTP (Roche Diagnostics). Following probe hybridization and washes, an Anti-DIG antibody conjugated to alkaline phosphatase (AP) (Roche) was incubated with embryos overnight at 4°C, and detection of AP activity was carried out using BM Purple (Roche).

Embryos were fixed overnight in 4% PFA and processed into paraffin wax. Sections were cut at a thickness of 5 µm. *In situ* hybridization was performed on paraffin sections (5 µm) according to the protocol from Chotteau-Lelievre et al. (2006) with minor modifications, and detection of alkaline phosphatase activity was visualized using BM Purple (Roche Diagnostics). Following staining, slides were quickly dehydrated in 80 % and then 100 % ethanol, cleared twice for 1 min in xylene (Roth) and coverslips were mounted with Entellan mounting medium (Merck). Images were captured using AxioVision software (Zeiss) with a Zeiss AxioCam. At least 2 embryos were analyzed and representative images taken.

Zebrafish (Zf) Lines and Maintenance

Zf were kept according to national law and to recommendations by Westerfield [2] in our fish facility in Bonn, Germany. Zf larvae of wild-type AB/TL and the transgenic strains

*Tg(wt1b:*eGFP*)* and *Tg(HGj4A)* were obtained by natural spawning and raised at 28°C on a 14 h light: 10 h dark cycle. Staging was performed according to Kimmel et al. [3].

Zf experiments with CRISPR/Cas9 were performed in Casper (*mitfa*$^{w2/w2}$*; mpv17*$^{a9/a9}$) at Boston Children's Hospital (BCH) Aquatic Core Facility. All national and institutional guidelines for the care and use of laboratory animals were followed. The zebrafish experiments were approved by the BCH Institutional Animal Care and Use Committee (IACUC).

Microinjections of Morpholino Oligonucleotides and mRNA

Embryos at the one-cell to two-cell stage were pressure injected into the yolk with Morpholino® oligonucleotide (MO) synthesized by GeneTools, LLC. Injections were carried out with 0.75 ng of *bnc2* MO (1.7nL/embryo) (5'-GAGCTTTCTCCTTCTTCTCCTCCTC -'3) and 0.75 ng of standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-'3). For human mRNA rescue experiments 30 - 100 pg of *in vitro* transcribed human *BNC2* mRNA was injected into the yolk of one-cell embryos. *BNC2* mRNA was transcribed from cDNA clone HsCD00399335 (Harvard Medical School) containing ENST00000380672.8 and from a custom-made cDNA clone (BioCat GmbH) containing ENST00000418777.5 using the mMESSAGE Machine T7 Kit (Ambion) and the Poly (A) Tailing Kit (Ambion) according to instructions.

Target Selection and sgRNA Generation

Single guide RNA (sgRNA) targets were selected using the CHOPCHOP online tool v1 following their ranking algorithm [4]and were generated by *in-vitro* transcription from

oligonucleotide based templates using the MEGAscript T7 Transcription Kit (Ambion). 2 µl of sgRNA stock (500 ng/µl) were mixed with 2 µl of recombinant Cas9 protein (1 µg/µl, PNA Bio, Thousand Oaks, CA) and incubated on ice for at least 10 min to allow formation of the sgRNA/Cas9 complex. 2 nl of the injection mix was injected intracellularly in one-cell stage zebrafish embryos. DNA was extracted and analyzed via Sanger sequencing to confirm mutagenesis. ICE synthego online tool was used to analyze sequences for insertions and/or deletions and for rate of mutagenesis (https://ice.synthego.com/#/).

Microinjections of Cell Tracer Dye CellTracker™ Red CMTPX

Tricaine (Sigma-Aldrich, Munich, Germany) at a concentration of 0.1–0.5 % (in 30 % Danieau's solution) was used to anesthetize larvae before microinjection of cell tracer dye CellTracker™ Red CMTPX (ThermoFisher Scientific) in the vesicle at a concentration of 5 uM. Larvae were consecutively imaged and kept up to 3 days post fertilization.

Western Blot Analysis

Zf samples were lysed in RIPA buffer with 4 % protease inhibitor, proteins separated by SDS-PAGE and transferred on PVDF membranes. Membranes were then incubated for several hours in blocking solution (5 % milk powder in TBST) before adding the anti-BNC2 primary antibody [2082C5a] in 1:1,000 dilution (Abcam) at 4°C overnight. After several washes in TBST the secondary HRP-goat anti-mouse antibody (Thermo Fisher Scientific) 1:10,000 dilution in blocking buffer was added for 1 hour at room temperature before imaging the membrane using enhanced chemiluminescent (ECL) HRP substrate for low-femtogram-level detection.

Whole-mount Zebrafish *In Situ* Hybridization (ISH)

Antisense and sense probes for *pax2a* were amplified from zebrafish poly-A embryonic cDNA and cloned in pBluescript. Antisense and sense probes for *bnc2* were amplified from cDNA clone IMAGE:7063814 (Source BioScience). Constructs were linearized by corresponding restriction enzymes and Dig-labelled RNA was synthesized using Roche Dig labelling kit. ISH was performed following instructions of Thisse et al., 2008[5].

Immunohistochemistry Staining of Whole-mount Zebrafish

Zebrafish larvae were euthanized using Tricaine (0.4–0.8 mg/ml) and fixed in 4% paraformaldehyde in PBS for 24h at 4°C. Samples were decalcified in 0.5 mM EDTA for 3 days at room temperature and embedded in paraffin according to standard procedures. Sections were obtained on a Leica RM2255 microtome (Leica Microsystems, Wetzlar, Germany). H&E staining was performed according to standard procedures. Immunohistochemical staining were performed with respective antibodies using Ventana Benchmark XT Automated IHC/slide staining system. Antibodies were used at the following dilutions. Anti-Cleaved caspase 3 (Cell signaling Technologies) 1:200 and Anti-BNC2 (Sigma) 1:200.

Imaging

At the stages of interest embryos were analysed under a Nikon AZ100 Macro-Zoom microscope and processed with NIS-Element Viewer software. Selected embryos were further anesthetized with 0.016 % tricaine, fixed in 2 % low-melting agarose and imaged

by two-photon scanning fluorescence in vivo microscope (LaVision Trim-ScopeII; Imspector and ImageJ software).

Statistical Analysis

Two-tailed Student's t-test, Mantel-Cox and two-way ANOVA test were used for analysis using GraphPad Prism version 6. Differences with a p-value of < 0.05 (*) were considered as being statistically significant.

Supplemental Web Resources

Human Splicing Finder, version 3.1 (www.umd.be/HSF3/)

ESE Finder, release 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home)

SFmap, version 1.8 (http://sfmap.technion.ac.il/)

Supplemental References

1. Yeo, G., and Burge, C.B. (2004). Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. J. Comput. Biol. J. Comput. Mol. Cell Biol. *11*, 377–394.

2. Westerfield, M. (2000). The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). (Eugene: Univ. of Oregon Press).

3. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev. Dyn. Off. Publ. Am. Assoc. Anat. *203*, 253–310.

4. Montague, T.G., Cruz, J.M., Gagnon, J.A., Church, G.M., and Valen, E. (2014). CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res. *42*, W401-407.

5. Thisse, C., and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. *3*, 59–69.

6. Shapiro, M.B., and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. *15*, 7155–7174.