Original research

X-linked variations in SHROOM4 are implicated in congenital anomalies of the urinary tract and the anorectal, cardiovascular and central nervous systems

Caroline M Kolvenbach,1,2 Tim Felger,1 Luca Schierbaum,3 Isabelle Thiffault,4 Tomi Pastinen,4 Maria Szczepańska,5 Marcin Zaniew,6 Piotr Adamczyk,7 Allan Bayat,8,9 Öznnur Yilmaz,1 Tobias T Lindenber, Holger Thiele,10 Friedhelm Hildebrandt,1,2 Katrin Hinderhofer,11 Ute Moog,11 Alina C Hilger,12,13 Bonnie Sullivan,14 Lauren Bartik,14 Piotr Gnyś,15 Phillip Grote,16,17 Benjamin Odermatt,1,18 Heiko M Reutter1,8, Gabriel C Dworschak1,3,19

ABSTRACT

Background SHROOM4 is thought to play an important role in cytoskeletal modification and development of the early nervous system. Previously, single-nucleotide variants (SNVs) or copy number variations (CNVs) in SHROOM4 have been associated with the neurodevelopmental disorder Stocco dos Santos syndrome, but not with congenital anomalies of the urinary tract and the visceral or the cardiovascular system.

Methods Here, exome sequencing and CNV analyses besides expression studies in zebrafish and mouse and knockdown (KD) experiments using a splice blocking morpholino in zebrafish were performed to study the role of SHROOM4 during embryonic development.

Results In this study, we identified putative disease-causing SNVs and CNVs in SHROOM4 in six individuals from four families with congenital anomalies of the urinary tract and the anorectal, cardiovascular and central nervous systems (CNS). Embryonic mouse and zebrafish expression studies showed Shroom4 expression in the upper and lower urinary tract, the developing cloaca, the heart and the cerebral CNS. KD studies in zebrafish larvae revealed pronephric cysts, anomalies of the cloaca and the heart, decreased eye-to-head ratio and higher mortality compared with controls. These phenotypes could be rescued by co-injection of human wild-type SHROOM4 mRNA and morpholino.

Conclusion The identified SNVs and CNVs in affected individuals with congenital anomalies of the urinary tract, the anorectal, the cardiovascular and the central nervous systems, and subsequent embryonic mouse and zebrafish studies suggest SHROOM4 as a developmental gene for different organ systems.

INTRODUCTION

SHROOM4, coding for Shroom Family Member 4, is a member of the Shroom protein family that contains a N-terminal PDZ domain, a coiled coil and a C-terminal ASD2 motif (figure 1A).1,2 Based on the domain structure, SHROOM4 protein may regulate the actin cytoskeletal architecture, which is critical for cell organisation during embryonic development.2 A wide range of cells show expression of Shroom4 during murine development, including the epithelium of the neural tube and kidney.1,2 It is further expressed in adult and fetal mouse brain structures, suggesting its implication in neural function and development.1,2

So far, single-nucleotide variants (SNVs) in SHROOM4 have been associated with Stocco dos Santos syndrome (MIM: 300434). Affected individuals show developmental delay (DD), mild-to-severe intellectual disability (ID), seizures, behavioural problems, autistic features, ataxia, short stature
Additionally, two individuals with moderate ID were found to have microduplications, microdeletions and complex rearrangements of 0.14–8.9 Mb in size comprising chromosomal region Xp11.22 with SHROOM4 (online supplemental table S1).8–15 Two recent studies report seven SNVs in SHROOM4 found in individuals with epilepsy.16 17 Furthermore, Heide et al18 found a nonsense variant in a fetus with corpus callosum agenesis. However, the phenotype of individuals with variations in SHROOM4 remains ill-defined.

We present six individuals from four families with congenital anomalies of the urinary tract and the anorectal, cardiovascular and central nervous systems (CNS), in whom exome sequencing and copy number variation (CNV) analyses detected rare and novel SNVs in SHROOM4, and microdeletions comprising SHROOM4, respectively. Embryonic mouse and zebrafish studies suggest the additional role of SHROOM4 in the development of the urinary tract and the anorectal and cardiovascular systems, in addition to the CNS.

METHODS

**Human subjects**

DNA was extracted from blood or saliva. Saliva samples were collected using the Oragene DNA self-collection kit (following the Oragene DNA Purification Protocol for saliva samples).

**Exome sequencing**

Exome sequencing was conducted in family A. The family was previously described by Hilger et al.19 For enrichment of genomic DNA, the NimbleGen SeqCap EZ HumanExome Library V2.0 enrichment kit was used. A 100 bp paired-end read protocol on an Illumina HiSeq 2000 sequencer was used. Filtering of mapped target sequences and data analysis were performed using the ‘Varbank’ exome analysis pipeline (https://varbank.ccg.uni-koeln.de/varbank2/) as described previously.20 In short, variants were ranked on the basis of their probable effect on the function of the encoded protein considering evolutionary conservation among orthologues across phylogeny using ENSEMBL Genome Browser (https://www.ensembl.org/index.html) and assembled using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), as well as the in silico prediction programmes PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (https://sift.bii.a-star.edu.sg/), Combined Annotation Dependent Depletion (CADD) (https://cADD.gs.washington.edu/) and Mutation Taster (http://www.mutationtaster.org/). Splice site variants were evaluated using the in silico prediction tools MaxEnt (http://hollywood.mit.edu/burgelab/mxamx/mxamxscan_scoreseq_acc.html), NNSPLICE (https://www.fruitfly.org/seq_tools/splice.html), SSF (http://www.umd.edu/searchsplicesite.html) and EX-SKIP (https://ex-skip.img.ca/cz/). Variant filtering based on population frequency was performed using population database gnomAD (https://gnomad.broadinstitute.org/) to include only rare alleles (ie, minor allele frequency <1%). Remaining variants were confirmed by Sanger sequencing. The website GeneMatcher (https://genematcher.org/) enabled the connection between researchers caring for family B.21 22

For family B, exome libraries were prepared using the Illumina TrueSeq PCR-Free library preparation kit with 10 cycles of PCR, followed by enrichment with the IDT xGen Exome Research Panel V2, with additional spike-in oligos (Integrated DNA Technologies) to capture the mitochondrial genome and dispersed genomic regions for CNV detection. Samples were sequenced to a minimum of 7 Gb of 2×125 paired-end reads for a mean of 80× average coverage or greater on the Illumina HiSeq 4000 or Illumina NovaSeq 6000. Bidirectional sequence is assembled, aligned to reference sequences based on human genome build GRCh37/UCSC hg19 and analysed using custom-developed software, RUNES and VIKING (https://www.childrensnery.org/childrens-mercy-research-institute/research-areas/genomic-medicine-center/data-and-software-resources/). The analysis is

Figure 1 Exome sequencing and copy number variation analyses in families with congenital malformations identify variants in SHROOM4. (A) Protein domain structures are depicted (https://smart.embl.de/). The position of newly identified single-nucleotide variants of families A and B and the partial deletion of SHROOM4 in families C and D are annotated in red. Previously reported single-nucleotide variants are shown in black. (B) Pedigree with two affected individuals in family A. III-1 presented with several congenital malformations with her maternally derived X-chromosome being activated in 84% of all lymphocytes. Pedigrees of families C and D that bear microdeletions of SHROOM4 and the partial deletion of SHROOM4 position of newly identified single-nucleotide variants are shown in black. Symbols representing affected individuals are shaded with different fill for differenting clinical conditions. Black boxes depict the congenital malformation phenotype. Striped boxes illustrate Dent’s disease. Brackets denote affection. Triangle denotes miscarriage. (C) Amino acid sequence conservation among species of p.Glu314Lys that was altered in families C and D that bear microdeletions including SHROOM4 and the partial deletion of SHROOM4. Embryonic mouse and zebrafish systems, in addition to the CNS.
confined to the genes of interest for the individual and with a
minor allele frequency corresponding to disease incidence.

Copy number variants
Initial diagnosis in individual II-2 (family C) was performed using
genome-wide oligonucleotide microarray (SurePrint G3
ISCA V2.8x60k, Agilent Technologies) with a mean resolution
of 60 kb. For molecular karyotyping of individuals II-1 and II-2
in family C, we used the Illumina Infinium Global Screening
Array-24 V2.0 plus multidisease add-on content BeadChip.
Visualisation of deletion was performed using GenomeStudio
Genotyping Module V2.0.5 (www.illumina.com/). Array anal-
ysis in family D was performed using the SurePrint G3 ISCA
V2.8x60k (Agilent Technologies). Detected CNVs in our study
were evaluated for overlapping CNVs annotated in the Data-
base of Genomic Variants (DGV) (http://dgv.tcag.ca) and in the
Database of genomic variation and Phenotype in Humans using
Ensembl Resources (DECIPHER) (https://decipher.sanger.ac.
uk/).

Skewed X-chromosome inactivation
Testing for skewed X-chromosome inactivation was performed in
individuals II-2 and III-1. We determined the X-inactivation
status at the CAG repeat of the first exon of androgen receptor
locus AR (NM_000044.4, DNA) was treated with methylation
specific restriction endonuclease HpaII. Untreated and untreated
DNAs were amplified by PCR with FAM-fluorescence labelled
HUMARA primer 1 (5’-GCTGTAAGGTTGCTTTCCCTCAT-3’) and primer 2 (5’-TCCAGATCTGTCAGAGCTG- GC-3’). PCR products were separated on an ABI PRISM 3100
Genetic Analyzer (Thermo Fisher). The fluorescence-labelled
PCR fragments were analysed with GeneMapper software
(Applied Biosystems). The peak area of each fragment was quan-
tified and compared between treated and untreated samples to
determine the methylation pattern of maternally and paternally
inherited X-chromosomes. Testing showed skewed X-inactiva-
tion in III-1 with her maternally derived X-chromosome being
activated in 84% of all lymphocytes.

Mouse in situ hybridisation (ISH)
Mouse embryos from a wild-type (wt) SWISS background of
embryonic days (E) 12.5 were dissected into PBS and fixed
overnight in 4% PFA at 4°C. Embryos were processed into
paraffin wax, and 5 μm sections were made using a microtome.
The probe corresponds to the 3’ coding region of Shroom4
(ENSMUSG00000068270). Two primers were used to amplify
a 995 bp region from mouse embryo cDNA (forward: TTGG-
GGCCCCAAGAAGGTC, reverse: TTCCCTGCACTC-
CACATGCT), and at the reverse primer, a T7 polymerase
sequence was included. The protocol of the probe generation
can be found online (http://mamep.molgen.mpg.de). In vitro
transcription was performed using a nucleotide mix containing
digoxigenin-11-UTP (Roche). All samples were processed for
ISH as described by Chotteau-Lelievre et al.24 with minor modi-
fications, and detection of AP activity was carried out using BM
Purrol (Roche). Images were obtained on a Leica M205C System
with a colour camera.

Zebrafish lines and maintenance
Zebrafish were kept according to national law and to recom-
mendations by Westerfield25 in our zebrafish facility in Bonn,
Germany. Zebrafish larvae (zfl) of wt AB/TI and the transgenic
Tg(act1:zeGFP) reporter lines were obtained by natural spawning
and were raised at 28°C in Danieu (30%) on a 14-hour light:10-
hours dark cycle. All zebrafish experiments were performed at ≤5 days post fertilisation (dpf). To suppress pigmentation,
0.003% 1-phenyl-2-thiourea was added to the Danieu solution
for respective zfl from 1 dpf onward. Staging was performed
according to Kimmel et al.26

Whole-mount zebrafish ISH
The cDNA plasmid for the preparation of antisense and sense
probes for shroom4 was generated by PCR from zebrafish poly-T
embryonic cDNA with specific primers (forward: CATCATtg-
cgctgctgactgtagataaagctg, reverse: CATCATgacggctggatcttgcg-
cgctgctg). The resulting PCR products were cloned into
pGEM-T Easy. Constructs were linearised by corresponding
restriction enzymes, and Dig-labelled RNA was synthesised
using Roche Dig labelling kit. ISH was performed following the
instructions of Thiese and Thiese.27

Microinjections of morpholino oligonucleotides (MOs) and
mRNA
Embryos at the one-cell to two-cell stages were pressure injected
into the yolk with a splice blocking MO synthesised by Gene-
tools, LLC. Injections were carried out with 1.27 ng of shroom4
MO (1.8 nL/embryo) (5’-ACATTGTGTGTTGCTTAC
CTTG-3’) and 1.27 ng of standard control (Crl) MO (5’-CTCTC
TTACCTGAGCTAAATTATA-3’) that targets transcripts
shroom4-201 (ENSDART000011542.5) and shroom4-202
(ENSDART0000170100.3). For human mRNA rescue experi-
ments, 75 pg of in vitro transcribed human SHROOM4 mRNA
was injected into the yolk of one-cell embryos. SHROOM4
mRNA was transcribed from cDNA clone H06D041016 (Source BioScience) containing NM_020717.4 using the mMS-
SAGE Machine T7 Kit (Ambion 1340M) and the Poly (A) Tailing
Kit (Ambion AM1350).

Western blot analysis
Zfl were pooled into samples of 20–30 and lysed in RIPA buffer
on ice with 4% protease inhibitor using a sonicator. Protein
sample (50 μg) was separated by sodium dodecyl-sulfate poly-
acrylamide gel electrophoresis (SDS-PAGE), transferred on PVDF
membranes and probed with a custom made anti-Shroom4 anti-
body (1:1000; polyclonal, rabbit AB2501, anti Aa 550–565,
C-SERFATNRNEIRQKK; thermo Fisher Scientific) at 4°C
overnight.

Sulforhodamine 101 (SR101) excretion assay
Excretion assay with 0.02 mM SR101 was performed on 5 dpf as
previously described by our group.28

Eye-to-head ratio
Zfl were phenotyped at 4 dpf using a ZEISS Stemi508 for
brightfield imaging. The time point of 4 dpf was chosen since
the phenotype was most prominent. The diameters of the eyes
were measured with NIS-Element Viewer software. To account
for variation and growth effects, eye size was normalised to head
(snout to otic vesicle).29 Zfl were anaesthetised with 0.03% tric-
aine and fixed in 1.25% low-

Phalloidin stainings
Phalloidin stainings were performed according to previous
reports.30 31 shroom4 MO-injected zfl were fixed at 5 dpf in 4% PFA overnight at 4°C and subsequently washed three times for
5 min in phosphate-buffered saline (PBS) with 0.1% Tween-20

Developmental defects

Kolvenbach CM, et al. J Med Genet 2022;0:1–10. doi:10.1136/jmedgenet-2022-108738

J Med Genet: first published as 10.1136/jmg-2022-108738 on 15 November 2022. Downloaded from http://jmg.bmj.com/ on September 15, 2023 by guest. Protected by copyright.
Developmental defects

(PBST) and 5 min in PBS-Tx (2% Triton X-100). Afterwards, zfl were incubated for 2 hours in PBS-Tx to allow permeabilisation. Stainings with 2 μg/mL tetramethyl rhodamine B isothiocyanate (Sigma Aldrich, P1951) were conducted overnight at 4°C. Stained zfl were briefly washed in PBS before applying DAPI (ACDBio, RNAscope DAPI, 320838) overnight at 4°C. Zfl were washed in PBS before imaging.

Imaging

At the stages of interest embryos were analysed under a Nikon AZ100 Macro-Zoom microscope. Selected embryos were anaesthetised with 0.016% tricaine, fixed in 2% low-osmolar formaldehyde (P渤A Solutions, 50,000), washed in PBS before imaging.

Table 1 Genetic and phenotypical overview of affected individuals with changes in SHROOM4

| Family | Family A | Family B | Family C | Family D |
|--------|----------|----------|----------|----------|
| Sex    | Male     | Male     | Male     | Male     |
| Chr. position (hg19) | chrX:g.50378133 | chrX:g.50378133 | chrX:g.50345632 | Xp11.23p11.22 |
| Inheritance | Maternally inherited (skewed X chr) | Maternally inherited | Maternally inherited | Maternally inherited |
| Nucleotide change | NM_020717.4: c.940G>A | NM_020717.4: c.3846G>A | NM_020717.4: c.940G>A | NM_020717.4: c.165G>C |
| Minimal region affected | chrX:g.49,369,600–50,447,320 | chrX:g.49,369,600–50,447,320 | chrX:g.49,369,600–50,447,320 | chrX:g.49,375,617–52,838,206 |
| CADD | 20.5 | 20.5 | NA | 2.8 |
| gnomAD allele frequencies (hom/hemi/het/wt) | 0/0/1/78,700 | 0/0/1/78,700 | Not reported | Not reported |
| Renal and GU | Unilateral renal agenesis | Unilateral renal agenesis | Renal dysplasia and microopenis | PUV, cryptorchidism, proteinuria, hypercalciuria and nephrocalcinosis (Dent's disease) |
| Neurological development | Hypotonia | DD and intellectual disability | DD, delayed speech and intraventricular bleeding | DD, increased muscular tone of lower limbs and epilepsy |
| Gastrointestinal | EA with fistula (Vogt type 3b) | EA with fistula (Vogt type 3b) | Gastric reflux and failure to thrive | Failure to thrive |
| Anorectal | ARM with vestibular fistula | ARM | ARM | ARM |
| Cardiac | Tetralogy of Fallot | AOSD and persistent foramen ovale | AOSD and persistent foramen ovale | AOSD and persistent foramen ovale |
| Skeletal | Aplasia of right radius bone and left preaxial polydactyly | Cleftcystically of the fifth finger and equinovarus | Cleftcystically of the fifth finger and equinovarus | Cleftcystically of the fifth finger and equinovarus |
| Craniofacial features | Overfolded helix, depressed nasal bridge, highly arched eyebrow, hypertelorism, micrognathia and posteriorly rotated ears | Prominent frontal tumours, depressed nasal bridge, hypertelorism, high arched palate and retinal thinning | Prominent frontal tumours, depressed nasal bridge, hypertelorism, high arched palate and retinal thinning | Prominent frontal tumours, depressed nasal bridge, hypertelorism, high arched palate and retinal thinning |
| Skin and hair | Mongolian blue spot and single transverse palmar creases | Atopic skin, oedema of the dorsal parts of the feet and lower legs, and periodic red colour of oedematous skin | Single transverse palmar crease | Single transverse palmar crease |
| Endocrine | Hypothyrosis | Hypothyrosis | Hypothyrosis | Hypothyrosis |
| Immune | Low C3/C4 and low immunoglobulins | Low C3/C4 and low immunoglobulins | Low C3/C4 and low immunoglobulins | Low C3/C4 and low immunoglobulins |
| Electrolytes | Hyponatraemia, hypocalcaemia, hypophosphataemia, hypokalaemia, hypocalcaemia and hypophosphataemia | Hyponatraemia, hypocalcaemia, hypophosphataemia, hypokalaemia, hypocalcaemia and hypophosphataemia | Hyponatraemia, hypocalcaemia, hypophosphataemia, hypokalaemia, hypocalcaemia and hypophosphataemia | Hyponatraemia, hypocalcaemia, hypophosphataemia, hypokalaemia, hypocalcaemia and hypophosphataemia |
| Other | Single umbilical artery, nuchal cord and sacral dimple | Fanconi syndrome, suspected shaken baby syndrome with subdural haemorrhage and retinal bleeding | Retinitis pigmentosa, ophthalmoplegia and inguinal hernia | Retinitis pigmentosa, ophthalmoplegia and inguinal hernia |

ARM, aneural malformation; AOSD, atrial septal defect; CADD, Combined Annotation Dependent Depletion; chr, chromosome; DD, developmental delay; EA, oesophageal atresia; GU, genitourinary; hemi, hemizygous; het, heterozygous; hom, homozygous; Mb, megabase; NA, not applicable; PUV, posterior urethral valve; wt, wild type.
wt allele in III-1 with her maternally derived X-chromosome being activated in 84% of all lymphocytes tested (figure 1B and table 1). Successive exome analysis prioritising X-chromosomal variants revealed a rare variant in SHROOM4 (NM_020717.4), detected in the affected individuals II-3 and III-1 and in the conducting mother (II-2) and maternal grandmother (I-2) (NM_020717.4:c.940G>A, p.Glu314Lys, ClinVar: SCV002498760), but not in the other family members I-1, II-1 and III-2 (figure 1A,B, and table 1). The p.Glu314Lys glutamate is conserved among vertebrates down to Xenopus tropicalis (figure 1C). Other vertebrates exhibit the amino acid aspartate, also acidic, at this respective position. Three out of four in silico prediction programmes rate the amino acid change as potentially damaging (CADD: 20.5, MutationTaster: disease causing, SIFT: deleterious, PolyPhen-2: 0.003 (benign)). One heterozygous female carrier in 178700 alleles (allele frequency 0.000005396) for this variant has been reported in the gnomAD online database (table 1). The variant does not reside in one of the two known functional protein domains (figure 1A). According to the standards and guidelines for the interpretation of sequence variants of the American College of Medical Genetics and Genomics (ACMG), we rated this variant as variant of uncertain significance (VUS).

Using the online tool GeneMatcher, we identified another male individual (family B, II-1) presenting with a sacral dimple, atrial septal defect, unilateral kidney dysplasia, bilateral clindacuity of the fifth finger, left-sided single palmar crease and pes equinovarus (table 1).22,23 Additionally, he showed dysmorphic craniofacial features, gastro-oesophageal reflux, hypotonia and failure to thrive (table 1). A hemizygous novel splice site variant in SHROOM4 was identified by exome sequencing (NM_020717.4:c.3942+1G>A, ClinVar: SCV002498761), but not in the other family members I-1, II-1 and III-2 (figure 1A,B, and table 1). The variant resides in the essential splice-donor site, likely causing retention of intronic DNA or splicing out of exons, possibly disrupting the ASD2 motif (figure 1A).24 Four in silico splicing programmes (MaxEnt, NNSPLICE, SSF and EX-SKIP) predict that the nucleotide change has a 100% impact on splicing. The variant was maternally inherited (I-2) (figure 1B and table 1). The mother had a medical history of depression and seizures; furthermore, her medical history describes a cardiomegaly of unknown origin with medical reports lacking. The mother died in the first year of the child’s life. The variant was rated as VUS according to the ACMG guidelines.

**Microdeletion Xp11.23p11.22 with loss of SHROOM4 and CLCN5**

Individual II-1 from family C presented with congenital anomalies and neurocognitive impairment (figure 1B and table 1). He showed DD, later ID, short stature, short limbs, failure to thrive, PUV and Dent’s disease (table 1). Array CGH analysis in individual II-1 identified a 1.07 Mb microdeletion at Xp11.23p11.22 spanning chrX:g.49,375,617–50,447,320 (hg19) (ClinVar: SCV002498763) (figure 1A). This microdeletion was maternally inherited.

In accordance with the ACMG standards and guidelines for interpretation and reporting of postnatal constitutional CNVs, identified microdeletions in families C and D were rated as CNVs of uncertain clinical significance. We did not detect any relevant overlap with CNVs annotated in DGV. However, there was one individual annotated in DECIPHER with a 382 kb maternally inherited hemizygous deletion covering the first exon and a large proportion of intron 1 of SHROOM4.13 This individual (434791) was noted to have global DD. Other entries comprising very large (>50 Mb) deletions or entries lacking phenotype information were considered as not relevant.

**Expression of Shroom4/shroom4 in murine embryos and zfl**

To study the expression of the canonical Shroom4 transcript (ENSMUST0000103005.10) in mouse embryos, we performed ISH studies, which showed expression in the brain and other neuronal tissues, vertebrae and genital tubercle at E12.5 (figure 2A,B). In order to further investigate the function of SHROOM4 during organ development, we used zebrafish as a model organism. BLAST analysis with human SHROOM4 identified a single zebrafish shroom4 orthologue (ENSDARG0000079900). To study the expression of shroom4 in zfl, we generated a labelled antisense RNA probe for ISH. In accordance with previous work, we detected strong expression of shroom4 in the head, brain, eyes, heart, pectoral fins, the intestine and cloacal region at 48 hours post fertilisation (hpf) (figure 2C–E) and 72 hpf (data not shown).14

**Knockdown (KD) of Shroom4 leads to reduced survival, pericardial effusion (PE), glomerular cysts, abnormal eye-to-head ratio and cloacal anomalies**

The similarity of the amino acid sequences between the human SHROOM4 and zebrafish Shroom4 proteins amounts to 73%. In order to study the phenotypic effect of a Shroom4 deletion in zebrafish, we applied a KD with a splice blocking MO that targets both existing transcripts, shroom4-201 (ENSDART0000011542.5) and shroom4-202...
Developmental defects

Figure 2  Shroom4/shroom4 is expressed during early mouse and zebrafish development. (A,B)ISH with Shroom4 probe on a sagittal section of a representative E12.5 (TS21) *Mus musculus*. Shroom4 expression is visible (in blue) in the head and neuronal tissues (black arrows), Vertebrae (black arrowhead), and genital tubercle (asterisks). Magnification (square in A) highlights the expression in the developing genitourinary tract (B). (C) Whole-mount ISH with an anti-shroom4 probe shows the expression of *shroom4* RNA (in blue) at 2 days post fertilisation in the head, brain, eyes, fins, heart, the intestine (black arrowheads) and cloacal region in *Danio rerio*. Sense controls did not show a staining (not shown). (D) The cloaca is highlighted by an arrow in the enlargement. (E) The expression of *shroom4* is highlighted by a white arrow in the brain and by a white arrowhead in the heart in the enlargement. Positive expression in the pectoral fins is marked by the white asterisk. Scale bars represent 1 mm (A), 10 µm (B) and 100 µm (C). ISH. In situ hybridisation.

(ENSDART00000170100.3) (online supplemental figure S2A). Efficiency of MO-induced KD was demonstrated by RT-PCR with a decrease of wt *shroom4* expression and presence of an alternative band without exon 2 for transcript *shroom4*-201 (online supplemental figure S2B-C). Similarly, RT-PCR for transcript *shroom4*-202 confirmed efficiency, showing an insertion of 41 intronic bp (online supplemental figure S2B,C). No change in eef1a1 (ENSDARG0000039502) expression was detected, serving as Ctrl (online supplemental figure S2C). Western blot analysis confirmed these findings on the protein level (figure 3A). Following the KD of Shroom4, we observed reduced survival of the MO-injected zfl (26%) compared with Ctrl MO-injected zfl (76%) at 5 dpf (figure 3B). Furthermore, MO-injected zfl displayed short body length, PE, a reduced eye and head size (eye-to-head ratio), as well as glomerular cysts and pronephric dilatation (figure 3C–H). PE was found in 71% of *shroom4* morphants but only in 13% of Ctrl larvae (figure 3C,D). The measured eye size was normalised to head length to account for variation in embryo size (figure 3E). This ratio was significantly lower in *shroom4* MO zfl (0.37) compared withCtrls (0.46) (figure 3F). To assess the impact of the Shroom4 KD on the kidneys and urinary tract, we used the transgenic Tg(*set1b:eGFP*) fluorescent reporter line, expressing GFP in the kidney during development (figure 3G,H).35 We observed the occurrence of glomerular cysts and dilatation of the pronephric ducts at 2 dpf in 52% of with MO-injected zfl but only in 13% of Ctrls (figure 3G,H). Rescue experiments that were conducted by co-injection of human wt *SHROOM4* mRNA together with *shroom4* MO resulted in statistically significant increased survival (58%) (figure 3B). The observed phenotype PE in MO-injected zfl could be rescued (MO+wt RNA: 51%) (figure 3C). The reduced eye size and head length could also be rescued by co-injection with human wt *SHROOM4* mRNA to a ratio of 0.42 (figure 3F). Nevertheless, the occurrence of glomerular cysts and pronephric dilatation could not be rescued (47%) (figure 3G). Overexpression of human wt *SHROOM4* mRNA did not cause increased mortality or any phenotypical changes (figure 3B,C,F,G).

On the analogy of the observed anorectal phenotypes in family A, we were interested in studying the impact of Shroom4 depletion on cloacal development in zfl. Therefore, we applied phalloidin stainings that mark actin filaments and allow visualisation of zebrafish cloacal morphology.30 31 In comparison with Ctrls, the *shroom4* MO-injected zfl exhibited a distension of the hindgut and dilated distal pronephric ducts, potentially due to distal obstruction caused by cloacal malformation (figure 4A,B). Next, we performed an SR101 excretion assay to confirm the potential hindgut anomalies in MO KD zfl. Zfl ingest SR101, a red fluorescent dye labelling the intestine, which enables examination of the opening of the cloaca and ensuing excretion of SR101 at 5 dpf. *shroom4* MO-injected zfl showed a significantly prolonged time to excretion and fewer numbers of excretion (figure 4C–F). In addition, no or just oscillating peristalsis was observed in *shroom4* morphants in contrast to Ctrls that all showed regular peristalsis of the intestine (figure 4G). These assays demonstrate the high abundance of cloacal opening defects in *shroom4* morphants resembling the anorectal phenotype in humans.

DISCUSSION

Previously, variations in *SHROOM4* have been associated with Stocco dos Santos syndrome (MIM: 300434), a neurodevelopmental disorder (online supplemental table S1).3–15 36 Here, we identified genetic variations of different size affecting *SHROOM4* in individuals with multiple congenital anomalies of the urinary tract, the anorectum and cardiovascular systems, and the CNS. The respective individuals presented with overlapping phenotypical features, including congenital anomalies of the urinary tract system (6/6), the CNS (4/6), the anorectum (2/6) and the cardiovascular system (2/6) (table 1). The two affected individuals of family A fulfil the clinical criteria of VATER/VACTERL association, defined by the presence of at least three of the following CFs: vertebral anomalies (V), anorectal malformations (A), cardiac defects (C), tracheo-oesophageal fistula and/or oesophageal atresia (TE), renal anomalies (R) and limb anomalies (L).15 Previously described affected individuals with ID and DD carrying variations in *SHROOM4* presented with features that concern anatomical structures affected by the VATER/VACTERL spectrum (online supplemental table S1).2–8 10–14 These comprised the heart in one individual, kyphosis and scoliosis, small hands, small feet, camptodactyly and clinodactyly (online supplemental table S1). Nevertheless, kyphosis and scoliosis may be secondary to a neurogenic genesis, and the described limb anomalies are observed in other syndromes and are not specific for VATER/VACTERL association (ie, small hands and feet).

Kolvenbach CM, et al. J Med Genet 2022;0:1–10. doi:10.1136/jmedgenet-2022-108738
Pathogenic variants in CLCN5 are associated with Dent’s disease, a renal proximal tubulopathy, characterised by proteinuria, hypercalcioria and hyperphosphaturia, kidney stones and, in some cases, kidney failure. Around 8% of pathogenic variants are deletions. Three of the affected individuals described here carry a deletion comprising CLCN5, causing the phenotype of Dent’s disease. However, all three individuals presented also with PUV (3/3), an anatomical obstruction of the urethra not associated with Dent’s disease. Additionally, all three individuals (3/3) show neurodevelopmental disorders, also not associated with pathogenic CLCN5 variations. Our human genetic data suggest that the SHROOM4 deletions may be implicated in

![Figure 3](image-url)

**Figure 3** Knockdown of Shroom4 leads to glomerular cysts, eye abnormalities and PE. (A) Western blot analysis demonstrates a protein decrease in shroom4 MO-injected zfl for Shroom4 (184 kDa, F1Q6C1), derived from transcript shroom4-201, in comparison with Ctrl MO-injected zfl. The protein product for transcript shroom4-202 is too small to be detected by Western blot analysis (11 kDa, B5DGK9). β-Actin serves as Ctrl and shows an equal amount of protein in both samples. (B) Quantification of survival (n=5); zfl injected with shroom4 MO show a significant reduction of survival rate at 5 dpf compared with Ctrl MO. Survival of shroom4 MO is significantly rescued by coinjection of human WT SHROOM4 RNA. No alterations in survival have been observed by solely injecting WT SHROOM4 RNA. (C,D) Quantification of PE (n=3) shows significant occurrence in shroom4 morphants, a phenotype, which could be rescued by co-injection of WT SHROOM4 RNA. Black arrowhead highlights PE observed in a zfl injected with shroom4 MO at 4 dpf. (E,F) Eye-to-head ratio of injected zfl at 4 dpf (n=4). Measurement of the eye (red line) and head (black line, distance between the snout to the otic vesicle) was performed as visualised (F). Injection of shroom4 MO significantly reduced eye-to-head ratio, while WT mRNA co-injection in shroom4 MO-injected zfl significantly rescues the phenotypical effect. (G,H) Zfl injected with shroom4 MO develop glomerular cysts (white arrowheads in H) and dilatation of the pronephric ducts (white asterisks in H) that could not be rescued by human WT RNA (n=4). Images from in vivo observation through fluorescence microscopy (dorsal view) in Tg(wt1b:GFP) were taken at 2 dpf. Scale bars represent 100 μm. Ctrl, control; MO, morpholino oligonucleotide; ns, nonsignificant; PE, pericardial effusion; wt, wild type; zfl, zebrafish larvae. p <0.05 (*), p <0.01 (**), p <0.001 (***).
the formation of urinary tract anomalies and the neurodevelopmental disorders in these individuals. Interestingly, microdeletions comprising CLCN5 and SHROOM4 have been previously associated with DD, ID, growth retardation, dysmorphic craniofacial features and Dent’s disease (online supplemental table S1).9 10

The observed phenotypes in shroom4 MO KD zfl resemble somehow the phenotypical spectrum observed in the affected individuals with genetic SHROOM4 variations reported here. The KD of Shroom4 led to decreased eye diameter and head size. The observation of a reduced eye-to-head ratio within the morphant zfl suggests a growth-independent effect. This assay has been previously used as a proxy to easily observe effects on neurogenesis, migration and patterning, showing the close relation of eye size and neurodevelopmental disorders in the zebrafish model. Embryonic expression data in mouse embryos and zfl implicate Shroom4 in early brain formation, implicating that the observed reduced eye-to-head ratio could represent the neurodevelopmental phenotypes in individuals with Stocco dos Santos syndrome, that is, microcephaly, ID and DD (online supplemental table S1).6 10 Further phenotypical features of shroom4 MO morphants show affection of the cardiovascular system displaying PE. However, PE might be a non-specific MO effect also observed in Ctrl zfl. Although MO-induced PE could be rescued by wt SHROOM4 mRNA coinjection, it remains uncertain if PE following Shroom4 KD resembles the cardiovascular phenotypes of families A and B.40 41

The formation of the pronephric cysts and dilated ducts in shroom4 KD zfl is consistent with the observed anomalies of the upper and lower urinary tract in the affected individuals (table 1). These also resemble the bilateral hydropnephrosis caused by an anatomical obstruction due to PUV observed in individual II-4 of family D (figures 3 and 4 and table 1). The dilatation of the pronephric ducts in zfl could also be secondary to the mechanical obstruction of the observed cloacal malformation. The phalloidin staining and SR101 assay frequently visualised cloacal opening defects of the hindgut among shroom4 MO KD zfl, resembling the ARM in the affected individuals (figure 4B,D, table 1). The complete absence or only partial presence of peristalsis could be due to mechanical obstruction or possibly to a malfunction in the visceral nervous system.

The observed phenotypes PE, reduced survival and reduced eye-to-head ratio were significantly rescued by the coinjection of shroom4 MO and human wt SHROOM4 mRNA (figure 3B,C,F). These rescue experiments highlight that the observed phenotypes in shroom4 MO KD zfl, which resemble several phenotypical features of the reported families, are each specifically caused by the depletion of Shroom4 but can be reduced by coinjection of human wt SHROOM4 mRNA. While coinjection of shroom4 MO and human wt SHROOM4 mRNA resulted in a non-significant reduction of glomerular cyst formation, it still underlines the specificity of our KD for Shroom4. Together, the phenotypical changes in shroom4 MO KD zfl and the observed expression of Shroom4 in embryonic mouse tissues suggest SHROOM4 to be implicated in the development of CNS and other principal structures, that are, the urinary tract and the anorectal and cardiovascular systems. Whereas our KD experiments leading to depletion of Shroom4 resemble the genetic situation of the individuals with SHROOM4 deletions reported here, our data cannot provide evidence beyond all doubts that the missense variant of family A is indeed responsible for the respective phenotype, as we have not directly tested the variant in family A in our MO KD rescue experiments.

Hence, while additional functional studies are warranted investigating the impact of SHROOM4 SNVs, our study suggests that the developmental role of SHROOM4 might be beyond its embryonic role in CNS formation, including an additional role in the development of several other organ systems.

Author affiliations
1Institute of Anatomy, Medical Faculty, University of Bonn, Bonn, Germany
2Department of Pediatrics, Boston Children’s Hospital, Harvard Medical School, Boston, Massachusetts, USA
3Institute of Human Genetics, Medical Faculty, University of Bonn, Bonn, Germany
4Genomic Medicine Center, Children’s Mercy Hospital, Kansas City, Missouri, USA
5Department of Pediatrics, Faculty of Medical Sciences, Medical University of Silesia in Katowice, Zabrze, Poland
6Department of Pediatrics, University of Zielona Góra, Zielona Góra, Poland
7Department of Pediatrics, Faculty of Medical Sciences, Medical University of Silesia, Katowice, Poland
8Department of Epilepsy Genetics and Personalized Medicine, Danish Epilepsy Centre, Dianalund, Denmark
9Institute for Regional Health Services, University of Southern Denmark, Odense, Denmark
10Cologne Center for Genomics (CCG), Faculty of Medicine, University Hospital Cologne, University of Cologne, Cologne, Germany
11Institute of Human Genetics, Heidelberg University, Heidelberg, Germany
12Department of Pediatrics and Adolescent Medicine, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany
13Research Center On Rare Kidney Diseases (RECORD), University Hospital Erlangen, Erlangen, Germany
14Division of Clinical Genetics, Children’s Mercy Hospital, Kansas City, Missouri, USA
15MedeRe Hospital, Department of Genetics, Lodz, Poland
16Institute of Cardiovascular Regeneration, Center for Molecular Medicine, Goethe University, Frankfurt am Main, Germany
17Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, Germany

Kolvenbach CM, et al. J Med Genet 2022;0:1–10. doi:10.1136/jmedgenet-2022-108738
Developmental defects

4 Stocco dos Santos RC, Castro NHC, Lilia Holmes A, Beçak W, Tackels-Dorn H, Lindsey CJ, Lubis HA, Stevenson RE, Schwartz CE, Holmes AL. Stocco DOS Santos X-linked mental retardation syndrome: clinical elucidation and localization to Xp11.3–q12.1. Am J Med Genet A 2003;118A:255–9.

5 Hagens D, Dubos A, Abdifi F, Barbi G, Van Zutven L, Hoelzenbein M, Tonnemper N, Moscone C, Frayn J-P, Cheilly J, van Bokhoven H, Gez C, Dolfius H, Ropers H-H, Schwartz CE, de Cassio Stocco Dos Santos R, Kalscheuer V, Hanauer A. Disruptions of the novel KIAA1202 gene are associated with X-linked mental retardation. Hum Genet 2006;118:578–90.

6 Lopes F, Barbosa M, Ameur A, Soares G, De Sá J, Dias AI, Oliveira G, Cabral P, Temudo T, Calado E, Cruz IF, Vieira JP, Oliveira R, Esteves S, Sauer S, Jonasson I, Sivonen A-C, Gyllensten U, Pinto D, Miceli P. Identification of novel genetic causes of Rett syndrome-like phenotypes. J Med Genet 2016;53:180–9.

7 Farwell KD, Shahmirzadi EL, Khechen D, Powis Z, Chao EC, Tippin Davis B, Baxter RM, Zeng W, Mroske C, Parra MC, Gandomi SK, Lu I, Li X, Lu H, Lu H-M, Salvador D, Ruble D, Lao M, Fischbach S, Wen J, Lee E, Elliott A, Dunlop CLM, Tang S. Enhanced utility of family-centered diagnostic exome sequencing with inheritance model-based analysis: results from 500 selected families with undiagnosed genetic conditions. Genet Med 2015;17:578–86.

8 Honda S, Hayashi S, Imoto I, Toyama Y, Okazawa H, Nakagawa E, Goto Y, Inazawa J. Copy-Number variations on the X chromosome in Japanese patients with mental retardation detected by array-based comparative genomic hybridization analysis. J Hum Genet 2010;55:590–9.

9 Armanet N, Metay C, Brisset S, Deschenes G, Pineau D, Petit FM, Di Rocco F, Gossens M, Tachtjian G, Labrune P, Tosca L. Double Xp11.22 deletion including SHROOM4 and CLCN5 is associated with severe psychomotor retardation and donor dementia. Mol Cytogenet 2015;8:8.

10 Danyel M, Suk ER, Raie V, Gelfer M, Knaus A, Horn D. Familial Xp11.22 microdeletion including SHROOM4 and CLCN5 is associated with intellectual disability, short stature, microcephaly and dent disease: a case report. BMC Med Genet 2019;12:6.

11 Nizon M, Andréau J, Rooyck C, de Blois M-C, Bourel-Poncet E, Bourgeois B, Route O, David A, Delobello D, Duban-Bedu B, Giuliano F, Goldenberg A, Grotto S, Hérion D, Karmous-Benayla H, Keren B, Lacombe D, Lapière J-M, Le Caquin C, Le Galloudec E, Le Merrer M, Le Moing A-G, Mathieu-Dramard M, Nusbaum S, Picson D, Pinson L, Raoul O, Río M, Romana S, Rouberie A, Colleaux L, Turleau C, Yekemski M, Nabnott R, Molan V. Phenotype-Genotype correlations in 17 new patients with an Xp11.23p11.22 microdeletion and review of the literature. Genet Med 2015;17:111–22.

12 Grams SE, Arigoopoulos B, Lines M, Chakraborty P, Mogwowan-Jordan J, Geraghty MT, Tsang M, Eswara M, Tezcan K, Adams KL, Link L, Himes P, Kostner D, Zand DJ, Stalker H, Driscoll DJ, Huang T, Rosenfeld JA, Li X, Chen E. Genotype-Phenotype characterization in 13 individuals with chromosome Xp11.22 duplications. Am J Med Genet A 2016;170:967–77.

13 Froyen G, Van Esch H, Bauters M, Hollander K, Frints SGM, Vermeersch JR, Devriendt K, Frayn J-P, Marynen P. Detection of genomic copy number changes in patients with idiopathic mental retardation by high-resolution X-array-CGH: important role for increased gene dosage of XLRN genes. Hum Mutat 2007;28:1034–42.

14 Iszie M, Froyen G, Devriendt K, de Ravel T, Frayn J-P, Vermeersch JR, Van Esch H. Sporadic male patients with intellectual disability: contribution: X-chromosome copy number variants. Eur J Med Genet 2012;55:577–85.

15 Dong Z, Chau MHK, Zhang Y, Dai P, Zhu X, Leung TY, Kong X, Kwock YK, Stankiewicz P, Cheung SW, Koy CW. Deciphering the complexity of simple chromosomal insertions by genome sequencing. Hum Genet 2021;140:361–80.

16 Roulier L, Verny F, Barcia G, Chenyal N, Desguere I, Colleaux L, Nabnott R. Exome sequencing findingnd unexpected mutations in 22 patients with myofibrillar-atomic epilepsy: is there a major genetic factor? Clin Genet 2019;96:254–60.

17 Bian W-J, Li Z-J, Wang J, Luo S, Li B-M, Gao L-D, He N, Yi Y-H. SHROOM Variants Are Associated With X-Linked Epilepsy With Features of Generalized Seizures or Generalized Discharges. Front Mol Neurosci 2022;15:862480.

18 Heide S, Spetchian M, Valence S, Buratti J, Mach C, Lejeune E, Olin V, Massimilo M, Lehal M, Moutout L, Moutan G, Walle C, Gandolfini S, Lebel M, Quenouille-Marigalt G, Chantot-Bastauret S, Milh M, Brelaite F, Pattes V, Gultaub L, Putoux A, Tsatsaris V, Struyf F, van den Bergh RM, Zeng W, Mroske C, Parra MC, Gandomi SK, Lu I, Li X, Lu H, Lu H-M, Salvador D, Ruble D, Lao M, Fischbach S, Wen J, Lee E, Elliott A, Dunlop CLM, Tang S. Enhanced utility of family-centered diagnostic exome sequencing with inheritance model-based analysis: results from 500 selected families with undiagnosed genetic conditions. Genet Med 2015;17:578–86.

9 Kolvenbach CM, et al. J Med Genet 2022;59:1–10. doi:10.1136/jmedgenet-2022-108738

REFERENCES

1 Hildebrandt JD, Soriano P. Shroom, a PDZ domain-containing actin-binding protein, is required for neural tube morphogenesis in mice. Cell 1999;99:485–97.

2 Yoder M, Hildebrandt JD. Shroom4 (Kaaa1202) is an actin-associated protein implicated in cilia and flagella organization. Cell Motil Cytoskeleton 2007;64:63–9.

3 dos Santos RC, Barretto OC, Noronaya K, Castro NH, Ferraz OP, Walter-Moura J, Vescio CC. Bečak W. X-linked syndrome: mental retardation, hip luxation, and G6PD variant [Gdr(+)Butantan]. Am J Med Genet 1991;39:133–6.

10 Kolvenbach CM, Dworschak GC, Frese S, Japp AS, Schuster P, Wenzlitschke N, Yilmaz E, Gyllensten U, Pinto D, Maciel P. Identification of novel genetic causes of Rett syndrome-like phenotypes. J Med Genet 2016;53:180–9.

11 Karmous-Chen Y, Tachdjian G, Labrune P, Tosca L, Double Xp11.22 deletion including SHROOM4 and CLCN5 is associated with severe psychomotor retardation and donor dementia. Mol Cytogenet 2015;8:8.

12 Gherardi E, Bourgois B, Boute LB, TP, HT, AH, HM, SR, and BH. The use of family-authorized, appropriate credit is given, any changes made indicated, and the use of family-authorized, appropriate credit is given, any changes made indicated, and the use of family-authorized, appropriate credit is given, any changes made indicated, and the use of

13 Gherardi E, Bourgois B, Boute LB, TP, HT, AH, HM, SR, and BH. The use of family-authorized, appropriate credit is given, any changes made indicated, and the use of family-authorized, appropriate credit is given, any changes made indicated, and the use of family-authorized, appropriate credit is given, any changes made indicated, and the use of

14 Gherardi E, Bourgois B, Boute LB, TP, HT, AH, HM, SR, and BH. The use of family-authorized, appropriate credit is given, any changes made indicated, and the use of family-authorized, appropriate credit is given, any changes made indicated, and the use of
Developmental defects

variants in BNC2 are implicated in autosomal-dominant congenital lower urinary tract obstruction. *Am J Med Genet* 2019;104:994–1006.

21. Sobrera N, Schiettecatte F, Boehm C, Valle D, Hamosh A. New tools for Mendelian disease gene identification: PhenoDB variant analysis module; and GeneMatcher, a web-based tool for linking Investigators with an interest in the same gene. *Hum Mutat* 2015;36:425–31.

22. Sobrera N, Schiettecatte F, Valle D, Hamosh A, GeneMatcher: a matching tool for connecting Investigators with an interest in the same gene. *Hum Mutat* 2015;36:928–30.

23. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of Hpall and Hhal sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 1992;51:1229–39.

24. Chotteau-Lellière A, Dolié P, Gofflot F. Expression analysis of murine genes using in situ hybridization with radioactive and nonradioactively labeled RNA probes. *Methods Mol Biol* 2006;326:61–87.

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

26. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development in the zebrafish embryo. *Int J Dev Biol* 1995;39:253–310.

27. Thiss C, Thise B. High-Resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 2008;3:59–69.

28. Rieke JM, Zhang R, Braun D, Yilmaz Ozmut, Japp AS, Lopes FM, Pleschka M, Hilger AC, Schneider S, Newman WG, Beaman GM, Nordenskjöld A, Ebert A-K, Promm M, Rösch WH, Stein R, Hirsch K, Schäfer F-M, Schmiedeke E, Boemers TM, Lacher M, Kluth D, Gosemann J-H, Anderberg M, Barker G, Holmdahl G, Läckgren G, Keene D, Cervellione M, Chotteau-Lellière A, Dolié P, Gofflot F. Expression analysis of murine genes using in situ hybridization with radioactive and nonradioactively labeled RNA probes. *Methods Mol Biol* 2006;326:61–87.

29. Emerson SE, St Clair RM, Waldron AL, Bruno SR, Duong A, Driscoll HE, Hallif BA, McFarlane S, Ebert AM. Identification of target genes downstream of semaphorin6A/PlexinA2 signaling in zebrafish. *Dev Dyn* 2017;246:539–49.

30. Parkin CA, Allen CE, Ingham PW. Hedgehog signalling is required for cloacal development in the zebrafish embryo. *Int J Dev Biol* 2009;53:45–57.

31. Baranowska Körberg I, Hofmeister W, Marklijung E, Cao J, Nilsson D, Ludwig M, Draaken M, Holmdahl G, Barker G, Reutter H, Vukojevic V, Clementson Kockum C, Lundin J, Lindstrand A, Nordenskjöld A. Wnt3 involvement in human bladder extrophy and cloaca development in zebrafish. *Hum Mol Genet* 2015;24:5069–78.

32. Dietz ML, Bernardiak TM, Vendetti F, Kielec JM, Hildebrand JD. Differential actin-dependent localization modulates the evolutionarily conserved activity of Shroom family proteins. *J Biol Chem* 2006;281:20542–54.

33. Firth HV, Richards SM, Bevan AP, Clayton S, Corpas M, Rajan D, Van Voren S, Moreau Y, Pettett RM, Carter NP. Decipher: database of chromosomal imbalance and phenotype in humans using Ensembl resources. *Am J Hum Genet* 2009;84:524–33.

34. Thiss B, Thise C. Fast release clones: a high throughput expression analysis. *ZFIN direct data submission*, 2004.

35. Perner B, Englert C, Bollig F. The Wils tumor genes wt1a and wt1b control different steps during formation of the zebrafish pronephros. *Dev Biol* 2007;309:87–96.

36. Piton A, Redin C, Mandel J-L. XLI-D causing mutations and associated genes challenged in light of data from large-scale human exome sequencing. *Am J Hum Genet* 2013;93:368–83.

37. Levitschenko EN, Mommens LAH, Bökenkamp A, Kroes NV. [From gene to disease; Dent’s disease caused by abnormalities in the CLCN5 and OCRL1 genes. *Ned Tijdschr Geneeskd* 2007;151:2377–80.

38. Lieske JC, Milliner DS, Beara-Lasic L. *Dent Disease. GeneReviews® [Internet]* Seattle, WA: University of Washington, Seattle, 2012: 1993–2021.

39. Novorol C, Burkhardt J, Wood KJ, Iqbal A, Rocque C, Coutts N, Almeida AD, He J, Wilkinson CJ, Harris WA. Microcephaly models in the developing zebrafish retinal neuroepithelium point to an underlying defect in metaphase progression. *Open Biol* 2013;3:130065.

40. Hanke N, Stagg L, Schroder P, Liljart J, Flieg S, Kaufeld I, Paul C, Haller H, Schiffer M. “Zebrafishing” for novel genes relevant to the glomerular filtration barrier. *Biomed Res Int* 2013;2013:1–12.

41. Gee HY, Ashraf S, Wan X, Vega-Warren V, Esteve-Rudd L, Lovric S, Fang H, Hurld TW, Sadowski CE, Allen SJ, Otto EA, Korkmaz E, Washburn J, Levy S, Williams DS, Bakaloglu SA, Zoltnitskaya A, Ozaltin F, Zhou W, Hildebrandt F. Mutations in EMP2 cause childhood-onset nephrotic syndrome. *Am J Hum Genet* 2014;94:884–90.