Urinary Tract Effects of *HPSE2* Mutations

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ABSTRACT

Urofacial syndrome (UFS) is an autosomal recessive congenital disease featuring grimacing and incomplete bladder emptying. Mutations of *HPSE2*, encoding heparanase 2, a heparanase 1 inhibitor, occur in UFS, but knowledge about the *HPSE2* mutation spectrum is limited. Here, seven UFS kindreds with *HPSE2* mutations are presented, including one with deleted asparagine 254, suggesting a role for this amino acid, which is conserved in vertebrate orthologs. *HPSE2* mutations were absent in 23 nonneurogenic neurogenic bladder probands and, of 439 families with nonsyndromic vesicoureteric reflux, only one carried a putative pathogenic *HPSE2* variant. Homozygous *Hpse2* mutant mouse bladders contained urine more often than did wild-type organs, phenocopying human UFS. Pelvic ganglia neural cell bodies contained heparanase 1, heparanase 2, and leucine-rich repeats and immunoglobulin-like domains-2 (LRIG2), which is mutated in certain UFS families. In conclusion, heparanase 2 is an autonomic neural protein implicated in bladder emptying, but *HPSE2* variants are uncommon in urinary diseases resembling UFS.

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Urofacial, or Ochoa, syndrome (UFS) is an autosomal recessive congenital disease.[1–7] Affected children have enuresis and incomplete bladder emptying associated with detrusor and bladder outlet dyssynergic contractions. ESRD, urosepsis and vesicoureteric reflux (VUR) can occur. UFS individuals have abnormal facial movements when crying and smiling. Biallelic mutations of *HPSE2* cause UFS.[4–7] *HPSE2* encodes heparanase 2,[8] which inhibits endo-β-D-glucuronidase activity of heparanase 1,[9] itself implicated in releasing growth factors from heparan sulfate proteoglycans.[10–12]

Since the first descriptions of *HPSE2* mutations in UFS in 2010,[4,5] only two further such families have been reported.[6,7] We here describe seven new UFS kindreds with *HPSE2* mutations, representing the largest series to date of genetically defined, overtly unrelated families. Their mutations and key clinical features are listed in Table 1. The Supplemental Material details their clinical histories and investigations, and Supplemental Figure 1 illustrates examples of facial grimacing and dysmorphic bladders. Considering these and previously published mutations[4–7] (Figure 1A), it is clear that pathogenic *HPSE2* mutations are found across the gene's coding region. Most (*i.e.*, nonsense or frameshift mutations) would cause loss of function, but a subset may generate abnormal proteins. In Family 2, the deleted amino acids in exon 3 correspond to the linker sequence in heparanase 1 cleaved to yield an active enzyme,[8–11] and an exon 3 deletion was previously reported in a Spanish child with UFS.[4] The in-frame deletion of asparagine 254 in Family 6 targets an amino acid conserved between human, mouse, and frog heparanase 2.[12] It is predicted to be nitrogen-linked glycosylated[13] (Figure 1A) and may direct endoplasmic

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reticulum processing of heparanase 2.[13] Mahmood *et al.*[7] reported a homozygous *HPSE2* mutation changing asparagine 543 to isoleucine. The asparagine, located in the carboxy-terminus, is also highly conserved between vertebrate orthologs,[12] but it is not predicted to be glycosylated. Its role needs further study.

Certain *HPSE2* mutations found in apparently unrelated UFS families might be explained by founder effects (*e.g.,* c.1465_1466del AA [p.Asn489-Profs*126] in Irish kindreds).[5] In this study, the c.457C>T (p.Arg153*) mutation was found in Families 2, 4, and 7 from Portugal, Turkey, and Kosovo, respectively, and was reported in Turkish UFS twins.[4] Despite sharing a mutation (c.1516C>T, p. Arg506*),[5] disease severity varied in Ochoa's Columbian UFS cohort[2] and some individuals were recognized as having UFS only after the syndrome was diagnosed in a relative. In this report, probands in Families 4 and 5 were part of the pediatric cohort 4C Study, all with renal insufficiency and a subset with urinary tract anomalies. In both families, the UFS diagnosis had not been considered before our genetic screen and, moreover, UFS in siblings was recognized only after diagnosis of the syndrome in probands. Thus, UFS is probably underrecognized, particularly when urinary tract features are mild. Such phenotypic variability may be due to environmental influences (*e.g.*, superimposed urosepsis) or genetic modifiers. Mutations of *LRIG2,* encoding leucine-rich repeats and immunoglobulin-like domains 2, exist in some UFS families lacking *HPSE2* mutations.[14] We excluded the possibility (data not shown) that probands in Families 4 and 5, who had severe kidney disease, also carried mutations of *LRIG2*.

Compound heterozygous missense *LRIG2* variants have been reported[14] in 1 of 23 individuals with non-neurogenic neurogenic bladder, which phenocopies[15] the UFS bladder. Moreover, primary nonsyndromic VUR is heritable in humans,[16,17] so perhaps primary VUR is associated with rare variants in genes such as *HPSE2*. *HPSE2* was sequenced and multiplex ligation-dependent probe amplification undertaken in 23 non-neurogenic neurogenic bladder individuals.[14] No mutations were detected. *HPSE2* was sequenced in probands from 193 United Kingdom and 246 Irish families with nonsyndromic VUR.[16,17] In one, a heterozygous novel variant (c.422_423insGCCCGG-p.Asp141delinsGluProGly [NM_021828.4]) was detected; he presented neonatally with urosepsis and unilateral VUR. A sister with unilateral VUR carried one copy of the variant, an in-frame deletion of Asp141 with insertion of three residues, GluProGly (Figure 1, B and C). The insertion lies between the equivalent sequence, which links the two heparanase 1 subunits,[8,9] and thus, by analogy, may functionally disrupt heparanase 2. Heterozygous missense variants with a minor allele frequency <1% (Supplemental Table 1) were not over-represented in patients (5 of 439 probands) versus European ancestry controls (38 of 6503 cases; P=0.2).

It has been speculated that UFS bladder defects are neurogenic because similar dyssynergia can occur after spinal cord damage.[2,18] Moreover, nerves invading human fetal bladders contain heparanase 2 and LRIG2.[14] At mouse embryonic days (E) 14 and 17 (Supplemental Figure 2 and data not shown), *Hpse2* transcripts were detected by RT-PCR in the bladder, metanephros, hindgut, neural tube, and eye. *Lrig2* RNA was detected in these locations and in the brain, heart, and liver. Pelvic ganglia give rise to postganglionic parasympathetic axons mediating detrusor contraction, while lumbar ganglia emit postganglionic sympathetic axons mediating detrusor relaxation and sphincter contraction.[19,20] Immunohistochemistry at E14 (Figure 2A) showed heparanase 2 in pelvic and lumbar ganglia; nerves running in bladder wall mesenchyme, beginning to form smooth muscle at this stage[21]; mesenchymal ureteric cells; and peripheral nerves within the head. The protein data accord with *Hpse2* transcripts being detectable in embryonic pelvic ganglia,[22] where *Hpse2*+ cells coexpress SRY-related HMG-box 10, which regulates neural crest development.[19] LRIG2 and heparanase 1 were also immunodetected in E14 pelvic and lumbar ganglia. Postnatally, bladder wall nerves and pelvic ganglia contained heparanase 1 and 2 and LRIG2 (Figure 2, B–I). Heparanase 1 elicits neuritogenesis in cell culture,[23] and heparanase 2 inhibits its enzymatic activity.[13] Thus, the *HPSE2* gene product may modify heparanase 1 activity within ganglia. Our observation that LRIG2 was also present in pelvic ganglia, combined with a report that LRIG2 modifies growth factor signaling in glioma cells,[24] suggests that LRIG2 may operate in similar biologic pathways as heparanase 2. If autonomic nerve function is perturbed, then the cyclic low-pressure storage of urine and complete bladder emptying will be impaired.[20] We speculate that this abnormal scenario operates in UFS. Here, there is an analogy with another congenital bladder disease in which M3, the cholinergic receptor mediating detrusor contraction, is mutated.[25]

Mating heterozygous mice carrying a gene trap in intron 6 of *Hpse2* led to birth of wild-type, heterozygous, and homozygous offspring in Mendelian ratios. Homozygous mutant tissue contained a truncated *Hpse2* transcript but lacked wild-type *Hpse2* transcripts extending beyond the trap (Figure 3A). Given that UFS is a congenital disease characterized by incomplete bladder emptying, we studied bladders in the first month of life when neural circuits normally become established.[26] At autopsy, we recorded whether bladders appeared as "full" translucent globes or "empty" opaque beads (Figure 3B). In the first fortnight after birth (Figure 3C), of 17 wild-type bladders, 4 were full and 13 empty; of 14 homozygous bladders, 13 were full and 1 empty (wild-type versus mutant; P<0.001, Fisher exact test, two tailed). In the second fortnight, all 15 wild-type bladders were empty; of 29 homozygous bladders, 14 were full and 15 were empty (P=0.001). Heterozygous bladders did not differ significantly from wild-types, and there was no influence of sex. Injecting India ink into homozygous bladders demonstrated urethral patency. In humans with UFS, large bladders have been reported

Table 1. Key clinical features and *HPSE2* mutations found in the seven families

Country (Parental Origin)	Family/Case, Sex	Bladder Phenotype	Upper Urinary Tract	Kidney Excretory Function	*HPSE2* Mutations
Saudi Arabia	1/a, M	Trabeculation	Antenatal unilateral HN; postnatal progressive unilateral HN	Normal at 1 yr	Homozygous c.57dupC (p.Ala20Argfs*45) generating premature stop codon at position 64
Portugal/Romani	2/a, F	Dysfunctional voiding and postvoiding residual	Normal	Normal at 5 yr	Homozygous apparent in-frame deletion of exon 3
	2/b, M	Dysfunctional voiding and postvoiding residual	Bilateral HN	Normal at 11 yr	
	2/c, F	NR	NR	NR	Compound heterozygous deletion of exon 3 and nonsense mutation c.457C>T (p.Arg153*)
Great Britain/America	3/a, F	Antenatal megacystis; postnatal trabeculation with dyssynergia in normal-capacity bladder	Unilateral focal kidney defect; no VUR	Normal at 14 yr	Compound heterozygous mutation. c.724delC (p.Leu242*) introducing a premature stop codon after a frameshift, and c.1099–1G>A leading to loss of splice acceptor and introduction of premature stop codon
Turkey	4/a, M	Trabeculation and incomplete emptying	Bilateral HN and bilateral focal kidney defects	Plasma creatinine raised (3.7 mg/dl at 12 yr)	Homozygous nonsense mutation c.457C>T (p.Arg153*)
	4/b, M	Pseudodiverticulae	Unilateral VUR	Normal at 11 yr	
Turkey	5/a, M	Trabeculation with postvoiding residual and high bladder pressure	Bilateral HN and bilateral focal kidney defects	Plasma creatinine raised (3.7 mg/dl at 9 yr)	Homozygous nonsense mutation c.429T>A (p.Tyr143*)
	5/b, M	Trabeculation and diverticulae	Normal	Normal at 13 yr	
Saudi Arabia	6/a, M	Non-neurogenic neurogenic bladder	Unilateral HN and renal parenchymal thinning	NR	Homozygous c.761_763del (p.[Asn254del]), an in-frame deletion of asparagine 254
Kosovo	7/a, M	Trabeculation and postvoiding residual. Low compliance.	Unilateral HN	Plasma creatinine raised (1.9 mg/dl at 6 yr)	Homozygous nonsense mutation c.457C>T (p.Arg153*)

M, male; HN, hydronephrosis; F, female; NR, not reported. See Supplemental Material for further clinical details.

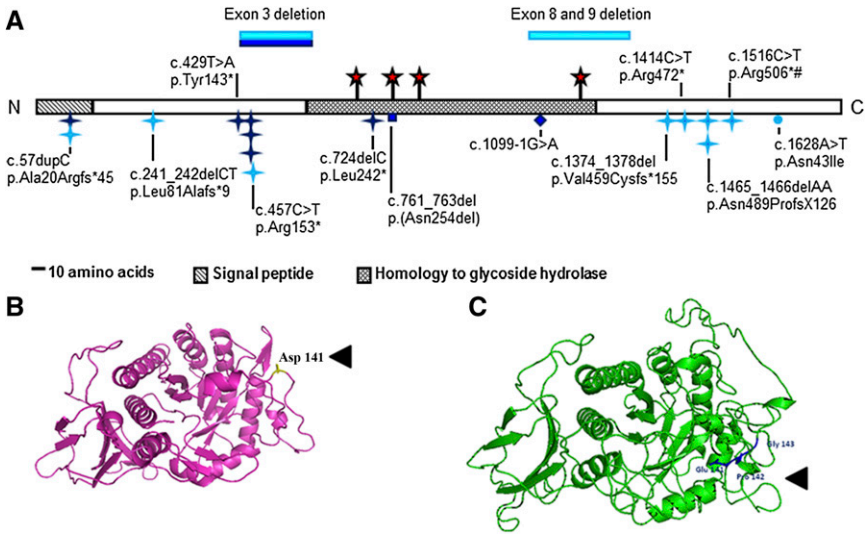

Figure 1. *HPSE2* mutations in UFS and the variant in primary VUR. (A) Schematic of heparanase 2 showing location of mutations. Dark blue indicates mutations described in this report. Light blue indicates mutations from previous reports. Blue stars, nonsense or frameshift mutations; circle, missense mutation; diamond, splice-site mutation; red stars, predicted N-glycosylation sites; #, founder mutation in Ochoa's Columbian cohort. Domains were predicted by Pfam and SignalP. N and C, the proteins amino and carboxy terminals, respectively. (B and C) Wild-type full length wild-type heparanase 2 protein (B) and the c.422_423insGCCCGG-p.Asp141delinsGluProGly variant (C). The heparanase 2 sequence was aligned to 51 α-L-arabinofuranosidase with ClustalW, and the structural model was generated using PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

antenatally and neonatally,[3] whereas bladder capacity may be normal later in childhood (*e.g.*, Family 3). Perhaps the change in bladder phenotype between the first and second postnatal fortnights in *Hpse2* mutant mice (93% versus 48% "full") reflects an analogous evolution of dysfunction in this species.

Homozygous mutant bladders contained muscle and urothelium (Figure 3, D and J). Quantitative PCR showed similar levels of epithelial (*uroplakin 3A*) and smooth muscle (*α-smooth muscle actin* and *myosin heavy chain 11*) transcripts in homozygous and wild-type littermate bladders harvested at 1 and 14 days after birth (Supplemental Figure 3). These results argue against a primary myogenic defect, as is present in the previously reported megabladder mutant mouse.[27] In turn, this supports the conclusion that the *HPSE2* gene product is necessary for peripheral neural control of bladder function. Heparanase 2 deficiency during *Xenopus* embryogenesis increases phospho-ERK (pERK),[12] so we immunoprobed mouse pelvic ganglia for pERK (Supplemental Figure 4). Although a subset of neural cell bodies immunostained for pERK, the patterns were similar in mutants and wild-type littermates. So, if heparanase 2 acts to modulate intracellular signaling in pelvic ganglia, it may do so by other means, and we note that the heparanase 1, itself antagonized by heparanase 2,[13] regulates AKT and SRC as well as ERK signaling.[10]

Homozygous mice showed poor weight gain in the third week, and subsequently some died. We performed autopsies in postnatal weeks 2–3. No pathologic abnormality was noted in the lungs. Homozygous kidneys were grossly normal, with a well defined cortex, outer medulla. and papilla (compare Figure 3, E–H, with Figure 3, K–N). These findings accord with the observation[12] that knockdown of heparanase 2 in embryonic frogs does not lead to overtly malformed pronephric kidneys. Although *Hpse2* mutant mouse kidneys lacked major aplastic/hypodysplastic malformations, three harbored focal cortical defects manifest as glomerular crowding (compare Figure 3, H and N) and/or tubular atrophy (compare Figure 3, I and O). Whether these are analogous to isotope scanning defects detected in some UFS kidneys (*e.g.*, Families 3–5), and which may result from ascending infection, remains to be established.

CONCISE METHODS

Human Genetic Studies

Since discovering mutations of *HPSE2*[4] and *LRIG2*[14] mutations in UFS, our research laboratory has sought mutations of these genes in other UFS families. Ethical approval was granted, and informed consent was obtained from all patients and/or their parents. Parental consent was obtained to show images of faces. Human studies were approved by institutional ethical review and approval (University of Manchester [06138] and National Health Service ethics committees [06/Q1406/52 and 11/NW/0021]). The VUR DNA samples from the United Kingdom were collected under National Research Ethics Service no. MREC/01/6/15. Irish VUR DNA samples were collected at Our Lady's Children's Hospital Crumlin and the National Children's Hospital, Tallaght, Dublin, Ireland. *HPSE2*[4] and *LRIG2*[14] were sequenced as previously described. Multiplex ligation-dependent probe amplification was carried out using MRC-Holland reagents and standard protocol with custom made probes (conditions available on request).

Splicing Experiment

In silico prediction performed using the Human Splice Finder (www.umd.be/HSF) predicted that c.1099–1G>A mutates the splice acceptor of exon 8 and that potential alternative splice acceptor sites exist at nucleotides c.1100 and c.1121 within exon 8. *HPSE2* is not expressed by lymphocytes, so we were unable to prove the alternate splicing by sequencing of constitutional cDNA. To assess effects of the missense mutation on splicing, we therefore performed a splicing assay using the pTB vector[28] to express mini-gene constructs of the mutated and wild-type *HSPE2* sequence in HEK293 cells. Briefly, exon 8 and approximately 500 bp of flanking sequence

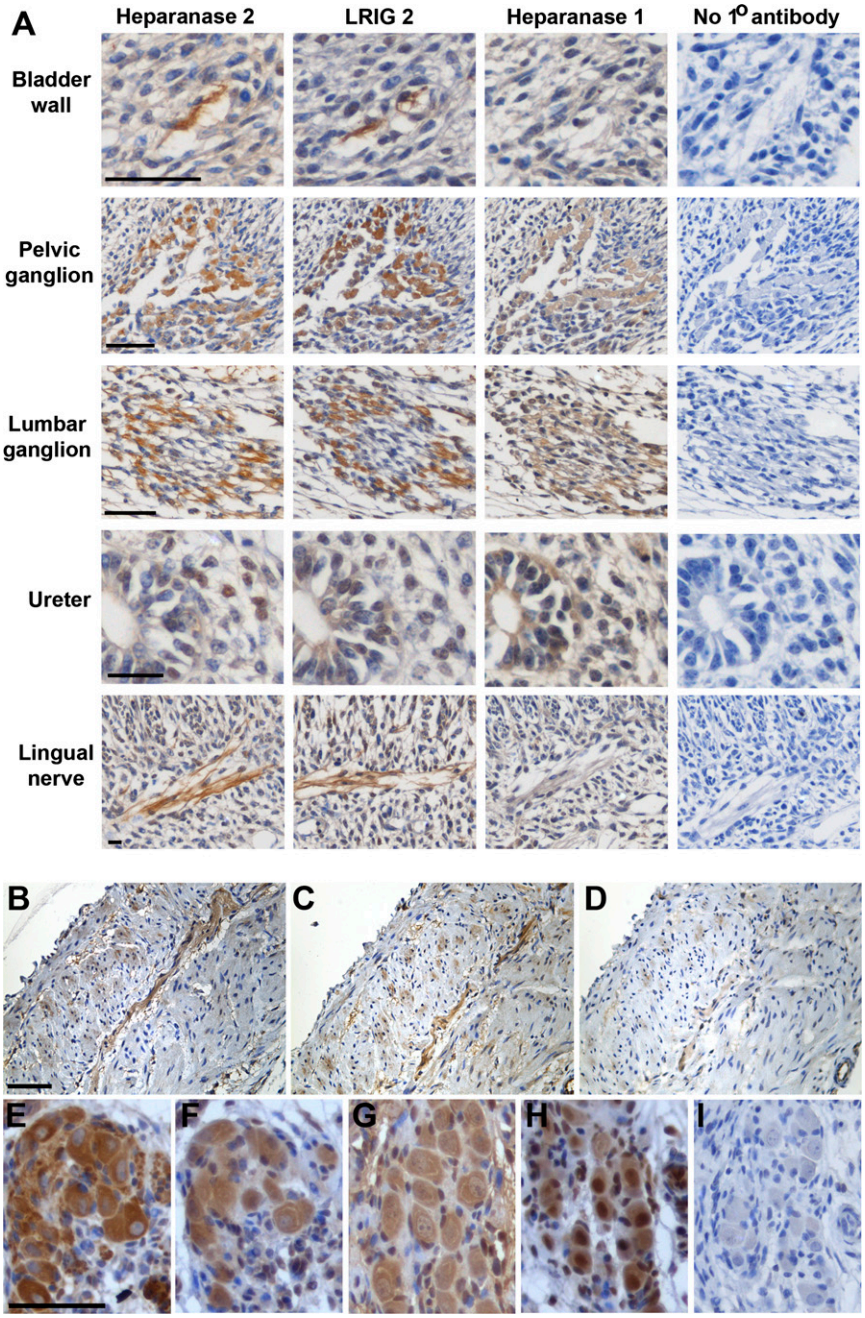

Figure 2. Immunohistochemistry in wild-type mice detects UFS proteins. Sections were counterstained (blue) with hematoxylin. Positive immunodetection signals are brown. (A) Embryonic day 14. Note the similar patterns for heparanase 2 and LRIG2 in a bladder wall nerve, a nascent pelvic ganglion, a nascent lumber ganglion, mesenchymal-like cells in the wall of the proximal (*i.e.*, top of the) ureter, and the lingual nerve. Heparanase 1 was also detected in the ganglia cell bodies but not in the nerve trunks themselves. (B–D) Serial sections of 1-week postnatal bladder immunostained for heparanase 2 (B) or LRIG2 (C). (D) The primary antibody was omitted. Note signals for both proteins in the nerve (running from top right to bottom left). (E–I) Immunohistochemistry of pelvic ganglia flanking the bladder outflow tract 2 weeks after birth: β3-tubulin, a neuronal marker (E); heparanase 2 (F); heparanase 1 (G); LRIG2 (H); negative control with no primary antibody (I). Scale bars are 50 μm.

were amplified with the oligonucleotide primers: forward 5′AAACATATGGGCTTTTAGGGAGTACGTGTGTCA-3′ and reverse 5′AACCATATGGCACTTCAGACAGCGGTAAGCAC3′ containing appended *NdeI* restriction sites. Amplicons derived from patient and control DNA were cloned into TOPO pCR2.1 vector and, following verification of correct sequence (BigDye Terminator v3.1Cycle Sequencing Kit, analyzed on the Applied Biosystems 3730xl DNA analyzer), were subsequently subcloned as *NdeI* digested fragments into the pTB vector using standard procedures. DNA of the mutant and wild-type expression constructs was prepared from bacterial cultures using the Qiagen mini-prep kit. For analysis of splicing, HEK293 cells cultured in low glucose DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin were transfected with the pTB mini-gene constructs using Effectene transfection reagents (Qiagen) according to the manufacturer's protocol. RNA was extracted 24 hours after transfection using the RNeasy kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using sequence specific oligo primers (5′TGTGCCTCCAGCTGAGGTGGT3′, internal exon 8 sequence and 5′GGTCACCAGGAAGTTGGTTAAATCA3′, pTB vector specific sequence) with Promega reverse transcription reagents according to a standard protocol. PCR amplification was performed using forward primer 5′CAACTTCAAGCTCCTAAGCCACTGC3′- with reverse primers 5′TGTGCCTCCAGCTGAGGTGGT3′ and 5′GGTCACCAGGAAGTTGGTTAAATCA3′.

PCR products were purified by gel extraction (EMD Millipore Montage DNA gel extraction kit) and sequenced as previously described. Two predominant variants were produced by expression of the mutant allele. The 5′ pTB exon was spliced into two alternate splice acceptors within exon 8, NM_021828.4 c.1100 and c.1121 (as predicted by Human Splice Finder). These both result in a frameshift that would be predicted to result in a premature stop mutation and nonsense mediated decay (p.Val367Glyfs*2 or p.Val367Lysfs*6).

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm were mounted on polylysine-coated glass

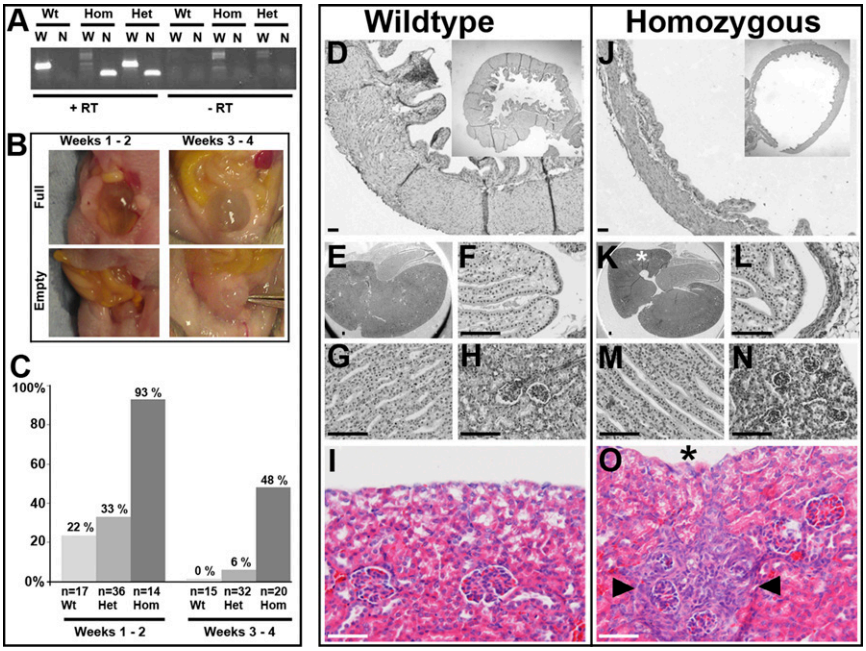

Figure 3. *Hpse2* mutant mouse bladder and kidney phenotypes. (A) RT-PCR of RNA from postnatal bladders. Note the abnormal *Hpse2* transcript (*N*, 158 base pairs) in gene trap homozygous (*Hom*) and heterozygous (*Het*) tissues. The wild-type *Hpse2* transcript (*W*, 364 base pairs) was the only one present in wild-type (*Wt*) mice but was not detected in homozygous (*Hom*) tissue. The right side of the gel shows the experiment when RT was not used. (B) Examples of "full" and "empty" bladder phenotypes in autopsies of mice in the first and second postnatal fortnights. (C) Frequencies of "full" bladder phenotypes in the three genotypes, in the first and second postnatal fortnights (total numbers analyzed are also shown). (D–O) Histology of mutant mouse bladders and kidneys; D–I are wild-type organs and J–O are homozygous mutant organs. All sections were stained with hematoxylin; I and O were also stained with eosin. Wild-type (D) and homozygous *Hpse2* mutant (J) bladders at 2 weeks. Note muscle in walls of both organs. When bladders are harvested, urine can escape and organs tend to deflate. Nevertheless, an impression of the difference between homozygous and wild-type bladders is shown in the low power insets (upper right of each frame). Wild-type kidneys at 2 weeks showing sagittal section overview (E) and higher powers of papilla (F), medullary tubules (G), and glomeruli (H). Counterpart zones in littermate homozygous kidney (K–N) are grossly similar to those in wild-type organ. An area of glomerular crowding in a mutant kidney is shown in N (from area marked by asterisk in K). High-power views of outer cortex in 3-week-old kidneys from wild-type (I) and mutant (O) littermate mice. Note the small area (demarcated by arrowheads in O) of glomerular crowding and tubulo-interstitial changes next to a concavity (asterisk) in the organ's surface. Scale bars are 50 μm.

slides. Endogenous peroxidase was quenched by incubation with hydrogen peroxide (30% solution in PBS). Antigen exposure was undertaken by heating at 95°C for 5 minutes in sodium citrate (pH, 6). Antibodies were used at optimized concentrations in PBS Triton-X (0.1%). Primary antibodies used were against β3-tubulin (AB9354; EMD Millipore), heparanase 1 (OBT1975G; AbD Serotec), heparanase 2 generated against NH2-QLDPSIIHDGWLDC-CONH2 (Generon) as validated by Roberts *et al.*,[17] LRIG2 (AP13821b; Abgent), and pERK (Sigma-Aldrich). Each was mixed with 3% serum specific to the species of the secondary antibody and incubated overnight at 4°C. Secondary antibodies were added for 2 hours at 4°C, followed by streptavidin-horseradish peroxidase (Vector) for 1 hour. 3, 3'-diaminobenzidine (Vector) was added for 2 minutes, and sections were counterstained with hematoxylin and/or eosin. For immunofluorescence, Alexa Fluor secondary antibodies (Life Technologies) were applied for 1 hour at room temperature, washed in PBS, and incubated with DAPI (Vector) for 10 minutes, washed in PBS, and mounted with Mowiol; a coverslip was then applied and sealed with nail varnish.

Detection of *Hpse2* and *Lrig2* Transcripts in Wild-Type Mice

Total RNA was extracted and following Dnase1 treatment, cDNA was generated by standard techniques. PCR was performed using Reddy Mix (Thermo Fisher Scientific) and the following primers: *Hpse2* forward 5′CTTAAGCTCCAAGCGTCTGG3′ and reverse 5′TGAATCCATCTAGGAGAGCAATG'3′; *Lrig2* forward 5′GTTATCGGCAGCAGGATGGA3′ and reverse 5′ACGAGGGTGTCTCTAACACTG3′; and *Gapdh* forward 5′GGGTTCCTATAAATACGGACTGC3′ and reverse 5′ CCATTTTGTCTACGGGACGA3′.

Mutant Mice

Mouse experiments were ethically approved by the University of Manchester Biologic Services Facility committee and the UK Home Office (PPL 40/3550, Modeling human UT malformations). *Hpse2* gene trap mice were initially studied on a mixed (129SvEvBrd×C57BL6N) background; subsequent experiments showed a similar bladder phenotype on a C3H background. Mutant founders were generated at the Texas A&M Institute of Genetic Medicine from embryonic stem cells infected with a replication-defective retrovirus (http://www.tigm.org/). The gene trap vector VICTR48[29] was found to have inserted into the sixth intron of *Hpse2*, which is located on chromosome 19 (Mouse accession NM_001081257 and Omnibank clone OST441123). The gene trap is predicted to cause splicing of exon 6 of *Hpse2* with inclusion of a *Neo* cassette and premature stop codon. Genotyping was carried out using a common forward primer (5′CCAGCCCTAATGCAATTACC3′) and wild-type (5′TGAGCACTCACTTAAAAGGAC3′) or gene trap (5′ATAAACCCTCTTGCAGTTGCA3′) reverse primers. RT-PCRs were undertaken to detect wild-type and gene trap insertion transcripts in postnatal bladders using the primers: *Hpse2* exon 5 forward 5′CGGACGGCCCAGGAAGAACG3′; *Hpse2* exon 9 reverse 5′TCCGTGGTCGAAAAATGAATGTC3′; and *Neo* reverse r5′TGTGCCCAGTCATAGCCGAATAGC3′. Samples were run on a 1% agarose gel with SafeView nucleic acid stain (NBS Biologic). After weaning,

homozygous mutant mice fail to gain weight and die when maintained for more than 1 month of age. The cause of their death is unknown. By contrast, heterozygous mice appear healthy; they are fertile and live to over 15 months of age. For quantitative PCR on mutant bladders, reactions were performed on a StepOnePlus platform (Applied Biosystems) and data analyzed using the accompanying software. The following Taqman probes (Applied Biosystems) were used: *Hprt1*, used as a reference housekeeping transcript (Mm00446968_m1); *Acta2* (Mm00656102_m1); *Myh11* (Mm00443013_m1); and *Upk3a*: (Mm00452321_m1).

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DISCLOSURES

None.

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Urinary tract effects of *HPSE2* mutations in humans and mice

SUPPLEMENTAL TEXT, TABLE AND FIGURES

Family histories and investigations

Family 1. The male proband has first cousin Saudi Arabian parents. Third trimester US detected left-sided hydronephrosis; it progressed in the first year and he received prophylactic antibiotics. Micturating cystourethrography (MCUG) showed bladder trabeculation without other anomalies, and plasma creatinine concentration (pCr) was normal. He grimaced from birth. Sequencing *HPSE2* revealed a homozygous frameshift in exon 1, c.57dupC (p.Ala20Argfs*45) [NM_021828.4], generating a premature stop codon at position 64. His asymptomatic parents were heterozygous.

Family 2. The female proband (V:3) is from a consanguineous Romani family living in Portugal (Supplemental Figure 1A and B). Antenatal ultrasound scans (USs) were normal but she grimaced from infancy. Aged three years, she experienced abdominal pain and urinary frequency. US revealed residual urine in a thick-walled bladder but no hydronephrosis. She was administered an α1 adrenergic antagonist to reduce outflow resistance and anticholinergics to reduce detrusor contractility. Aged five, she had nocturnal enuresis and normal pCr. Her paternal male cousin (V:1) also grimaces. He experienced lumbar pain and dysuria when nine years. US revealed a large thick-walled bladder and bilateral hydronephrosis; urodynamics showed dyssynergia. He self-catheterized and received anticholinergics. US at 11 years showed no hydronephrosis but the bladder remained abnormal. Polymerase chain reaction (PCR) failed to amplify *HPSE2* exon 3 in V:3 and V:1. Multiplex ligation-dependent probe amplification (MLPA) and gap PCR showed both had a homozygous in-frame deletion of 11.2 kb encompassing exon 3. IV:1 (mother of V:1),

IV:3 (father of V:3) and III:4 (paternal grandmother of V:1 and V:3) were heterozygous and had no urinary symptoms. III:4, however, and two of her paternal cousins (III:1 and III:2) grimace. III:4 carries a second *HPSE2* mutation, c.457C>T (p.Arg153*) [NM_021828.4] which would cause nonsense-mediated decay; DNA was not analysed in III:1 and III:2.

Family 3. The female proband has white British/American parents. Second trimester US detected megacystis and postnatal US revealed bladder wall thickening. MCUG showed a fir tree-shaped, trabeculated bladder; VUR was absent. At six years she was enuretic and urodynamics showed a normal capacity dyssynergic bladder. Dimercaptosuccinic acid isotope scanning (DMSA) identified a left kidney focal defect. Continence was established at nine years following α1 adrenergic antagonist administration. pCr was normal at 14 years. Examination by a Neurologist reported grimacing upon smiling, diminished central forehead and lateral eyebrow movements, and an inability to seal the lips. Sequencing *HPSE2* revealed novel compound heterozygous mutations, c.724delC (p.Leu242*) and c.1099-1G>A [NM_021828.4]. The former introduces a premature stop codon after a frameshift and the latter leads to loss of a splice acceptor and introduces a premature stop codon (see ***Splicing Experiment*** in **Methods**). The asymptomatic father was heterozygous for c.1099-1G>A.

Family 4. The male proband (Supplemental Figure 1C and D) from a consanguineous Turkish family presented with urosepsis aged two years when: US revealed bilateral hydroureteronephrosis; MCUG showed a large, incompletely-emptying trabeculated bladder but no VUR; and DMSA showed bilateral focal defects. Diurnal enuresis was managed by intermittent urethral catheterization. He had recurrent urosepsis. pCr was elevated at three (1.7mg/dl; normal <1.0mg/dl) and 12 (3.7mg/dl) years. *HPSE2* sequencing revealed a homozygous nonsense mutation, c.457C>T (p.Arg153*) [NM_021828.4], with heterozygous asymptomatic parents. Only then was it realized that he had a typical UFS grimace. His

younger brother (Supplemental Figure 1E and F) was homozygous and also grimaces. Aged eight years, he had primary nocturnal enuresis and MCUG revealed bladder pseudodiverticulae and unilateral VUR; pCr was normal.

Family 5. The male proband from a consanguineous Turkish family presented at four years with diurnal enuresis and urosepsis. US revealed bilateral hydroureteronephrosis, urodynamics demonstrated high intravesical pressures, MCUG showed a large trabeculated bladder without VUR, and DMSA showed bilateral focal defects. His used intermittent catheterization *per* urethra. pCr was elevated (3.7mg/dl) at nine years. Sequencing *HPSE2* revealed a novel homozygous nonsense mutation, c.429T>A (p.Tyr143*) [NM_021828.4]; only then was his characteristic grimace recognised (Supplemental Figure 1G). His asymptomatic parents are heterozygous but his brother grimaces (Supplemental Figure 1H) and is homozygous; at 11 years, he has primary nocturnal enuresis. MCUG demonstrated bladder trabeculation but no VUR, and pCr was normal.

Family 6. The proband is a 17 year male with third cousin Saudi Arabian parents. Antenatal US was not undertaken and he grimaced as a young child. He had a NNNB and left-sided hydroureteronephrosis with renal parenchymal thinning. He underwent bladder augmentation and catheterized using a Mitrofanoff stoma. Examination by a Neurologist revealed facial weakness with abnormal movement of the mouth and eyes with expression. He had a shuffling gait with low muscle bulk in his legs. Brain magnetic resonance imaging was normal. Sequencing revealed a homozygous *HPSE2* variant, c.761_763del [NM_021828.4], an in-frame deletion of asparagine 254. Family samples were unavailable.

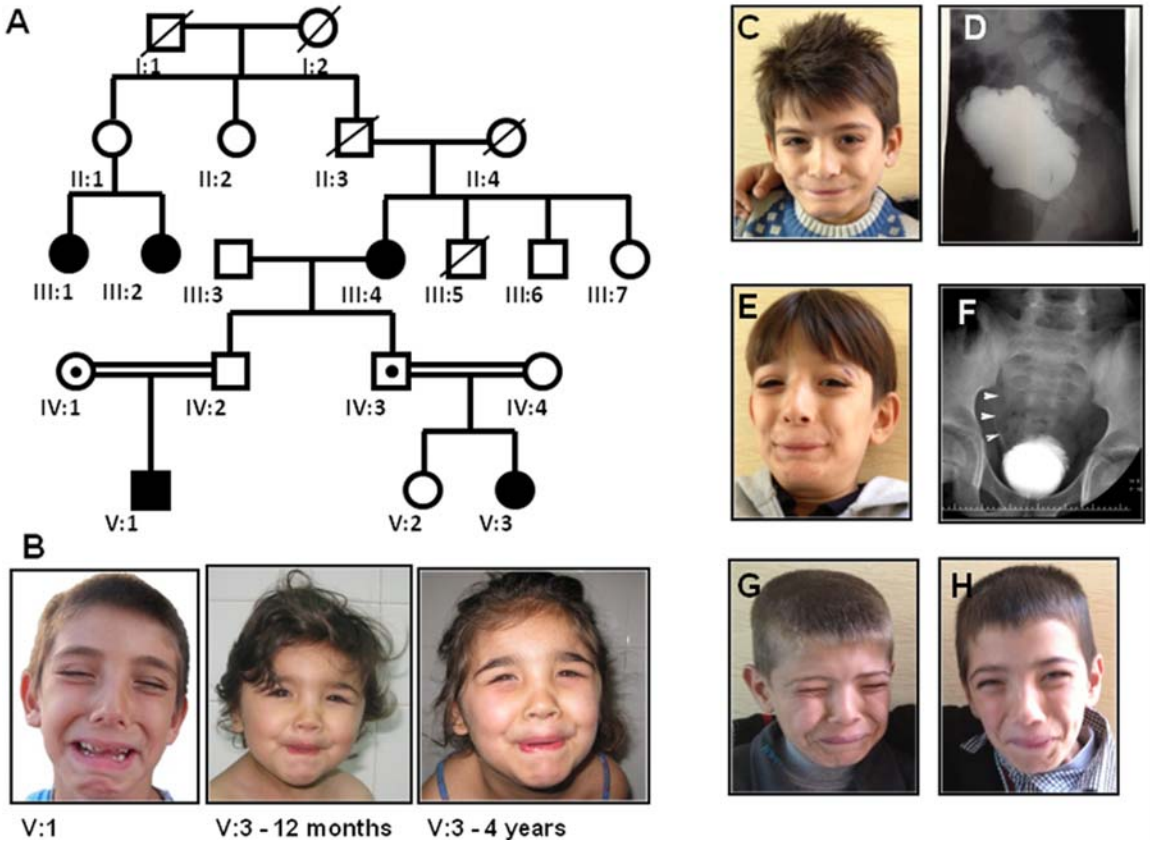
Family 7. The male proband from Kosovo presented with primary diurnal and nocturnal enuresis and urosepsis aged seven years. Investigations revealed bilateral hydroureteronephrosis, the left kidney contributing 39% of total function. Cystoscopy showed

a trabeculated bladder with outlet hypertrophy, and urodynamics showed high intravesical pressures and residual urine. Aged nine years, the left ureter was reimplanted when pCr was 0.7mg/dl. Bladder dysfunction and enuresis persisted. pCr was 1.39mg/dl at 13 years when self-catheterization *via* an umbilical Mitrofanoff stoma was initiated. Nevertheless, kidney function deteriorated with pCr 1.94mg/dl at 16 years. He grimaces when smiling. Sequencing *HPSE2* revealed the homozygous nonsense mutation, c.457C>T [NM_021828.4] (p.Arg153*).

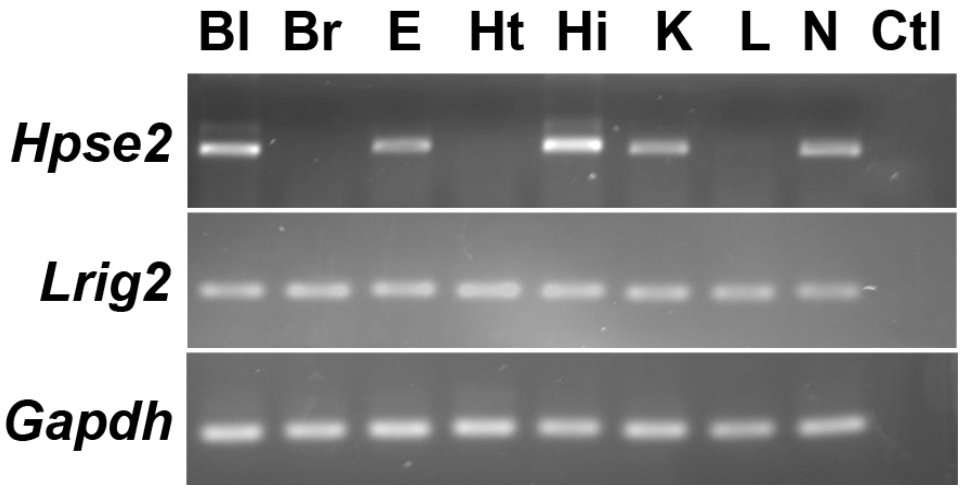
Supplemental Table 1. Rare (<1%) heterozygous missense variants in *HPSE2* in familial VUR cohorts.

Variant	Segregation of variant with VUR phenotype	Frequency in Variome Databases	Predicted Consequence
c.980C>A, p.Thr327Lys	Seen in three affected siblings and an apparently unaffected mother.	None	Align GVGD - Class C0 – less likely SIFT – Deleterious score 0.04
c.1021C>T, p.Arg341Trp	Seen in two affected female siblings, an apparently unaffected sister and their apparently unaffected father whose brother had VUR and a nephrectomy.	Not in databases. Adjacent to variant c.1022G>A, p.Arg341Gln causing change in same amino acid on ESP (0.03%).	Align GVGD – Class C65 – most likely SIFT – Deleterious score 0.00
c.1258C>T, p.Arg420Trp	No	Reported once in European American chromosomes on ESP (0.008%).	Align GVGD – Class C65 – most likely SIFT – Deleterious score 0.00
c.1618G>C, p.Val540Leu	No	dbSNP - rs140066668, Present in ESP (0.3%).	Align GVGD – Class C0 – less likely SIFT – Deleterious score 0.00
c.1769G>A, p.Arg590His	No	dbSNP - rs138098027. Present in ESP (0.01%)	Align GVGD – Class C0 – less likely SIFT – Deleterious score 0.00

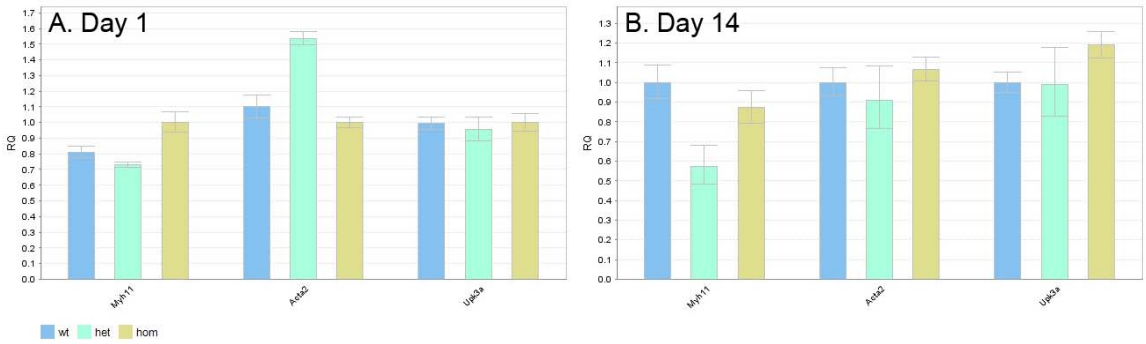
Legend: Each variant has only been seen once. Databases checked are dbSNP build 137 and NHLBI Exome Sequencing Project (ESP) Exome Variant Server. Prediction software used Align GVGD - http://agvgd.iarc.fr/, SIFT - http://sift.jcvi.org/, Human Splicing Finder - http://www.umd.be/HSF/



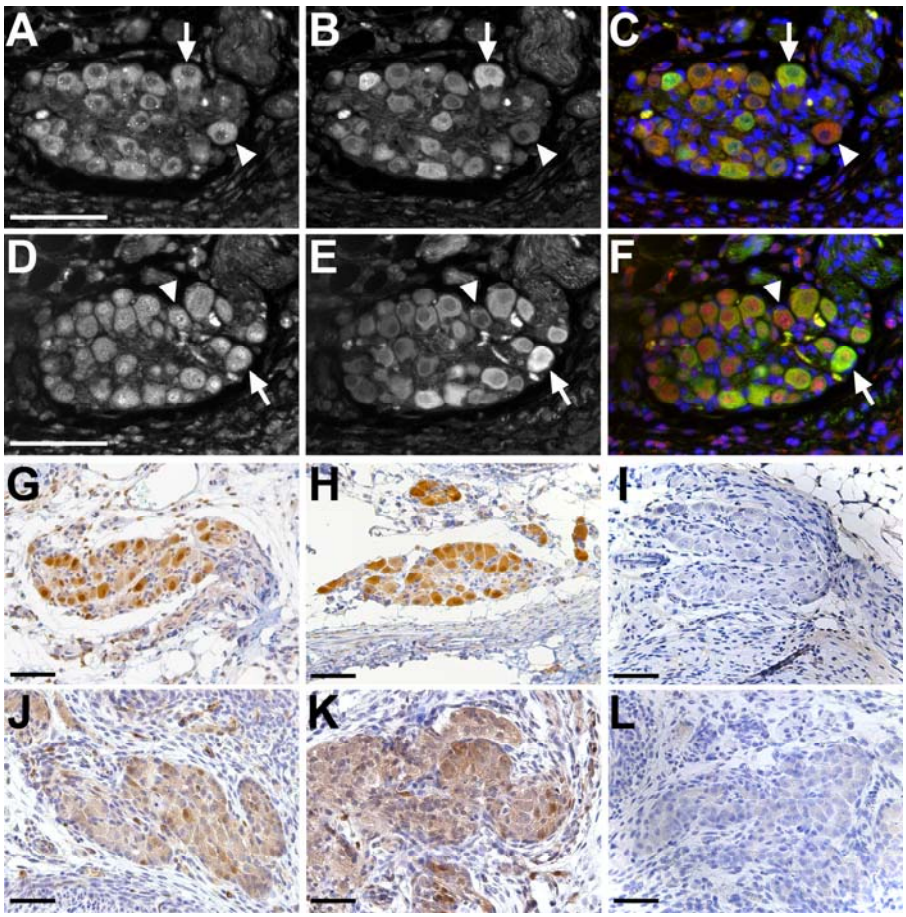
Supplemental Figure 1. Clinical details of selected families. A. Pedigree of Family 2. **B.** Faces of V:1 and V:3 from Family 2 showing grimace upon smiling. Note the simultaneous contraction of the corners of the mouth and eyes. **C.** Face of proband from Family 4. **D.** MCUG of proband from Family 4; note bladder trabeculation and pseudodiverticulae. **E.** Face of younger brother from Family 4. **F.** MCUG showing unilateral VUR (white arrowheads). **G.** Face of proband from Family 5. **H.** Face of affected sibling from Family 5



Supplemental Figure 2. RT-PCR analyses of wild-type E17 mouse organs. Note *Hpse2* expression in urinary bladder, eye, hindgut, kidney and neural tube. *Lrig2* was detected in all organs examined. *Gapdh* is included as a housekeeping control. Key: Bl, urinary bladder; Br, brain; E, eye; Ht, heart; Hi, hindgut; K, metanephric kidney; L, liver; N, neural tube; and Ctl, negative control with no cDNA.



Supplemental Figure 3. Quantitative PCR of bladder transcripts. RNA was extracted from whole bladders and smooth muscle *(Myh11* and *Acta2)* and epithelial *(Upk3a*) transcripts were quantified. Their levels are depicted after factoring for *Hprt1*, used as a reference housekeeping transcript. Note that levels of the muscle transcripts were similar in homozygous mutant (hom) compared with wild-type (wt) littermates at postnatal Days 1 and 14, thus providing no support for a myogenic pathogenesis of the bladder phenotype. Results are shown as average values, and the bars are SDs, relating to three replicate measurements of each transcript.



Supplemental Figure 4. Phospho-ERK in pelvic ganglia. Longitudinal histology sections through pelvic ganglia in two week old (**A-I**) and two day old (**J-L**) mice. **A-F.** A ganglion was imaged by immunofluorescence. **A**. Immunostaining for heparanase 2 (white) shows neural cell bodies containing the protein; **B.** same section immunoprobed for pERK (white) detects this protein in a subset of neural cell bodies; and **C.** merged image with all nuclei stained blue using DAPI, heparanase 2 in red and pERK in green. Note that some cells, such as the one indicated by the arrowhead, were positive for heparanase 2 but not pERK, whereas others, such as the one indicted by the arrow, contained both proteins. **D**. Immunostaining for LRIG2 (white) shows neural cell bodies containing the protein; **E.** same section immunoprobed for pERK detects this protein (white) in a subset of neural cell bodies; and **F.**

is a merged image with all nuclei stained blue using DAPI, the LRIG2 red and pERK in green. Note that certain cells (arrowhead) were positive for LRIG2 but not pERK, whereas others (arrow) contained both proteins. **G-I.** Brightfield images of pelvic ganglia with nuclei stained blue with hematoxylin. Two week old wild-type ganglion (**G**) immunostained for pERK (brown) shows a similar pattern to that of a homozygous *Hpse2* mutant ganglion (**H**). **I.** Two week old wild-type ganglion without primary antibody. **J-L.** Images of pelvic ganglia from two day old mice; wild-type immunostained for pERK (**J**); homozygous mutant immunostained for pERK (**K**); wild-type ganglion without primary antibody (**L**). Note pERK immunoreactivity is more diffuse than at two weeks of age, with similar appearances of wild-type and mutant tissues. Scale bars are 50 μm.