A founder mutation in EHD1 presents with tubular proteinuria and deafness

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Abstract: Background:
The endocytic reabsorption of proteins in the proximal tubule requires a complex machinery and defects can lead to tubular proteinuria. The precise mechanisms of endocytosis and processing of receptors and cargo are incompletely understood. EHD1 belongs to a family of proteins presumably involved in the scission of intracellular vesicles and in ciliogenesis. However, the relevance of EHD1 in human tissues, in particular the kidney, was unknown.

Methods:
Genetic techniques were used in patients with tubular proteinuria and deafness to identify the disease-causing gene. Diagnostic and functional studies were performed in patients and disease models to investigate the pathophysiology.

Results:
We identified six individuals (5-33 years) with proteinuria and a high-frequency hearing deficit associated with the homozygous missense variant c.1192C>T (p.R398W) in EHD1. Proteinuria (0.7-2.1 g/d) consisted predominantly of low-molecular-weight proteins, reflecting impaired renal proximal tubular endocytosis of filtered proteins. Ehd1 knockout and Ehd1R398W/R398W knockin mice also showed a high-frequency hearing deficit and impaired receptor-mediated endocytosis in proximal tubules, and a zebrafish model showed impaired ability to reabsorb low-molecular-weight dextran. Interestingly, ciliogenesis appeared unaffected in patients and mouse models. In silico structural analysis predicted a destabilizing effect of the R398W variant and possible inference with nucleotide-binding leading to impaired EHD1 oligomerization and membrane remodeling ability.

Conclusion:
A previously unrecognized autosomal recessive disorder characterized by sensorineural deafness and tubular proteinuria is caused by a homozygous missense variant in EHD1. Recessive EHD1 variants should be considered in individuals with hearing impairment, especially if tubular proteinuria is noted.
Significance Statement

Renal tubular protein reabsorption has been the focus of interest in the kidney community, and despite numerous associated inherited diseases, the detailed molecular basis remains poorly understood. Based on six patients with tubular proteinuria and sensorineural hearing deficit, EHD1 was identified as a critical component of the renal protein reabsorption machinery and inner ear function. As a key player in vesicular dynamics, EHD1 has previously been associated with early ciliogenesis. However, no obvious defect of ciliogenesis was found in the kidney of either the patients studied here or in knockin and knockout mice. In summary, these data may contribute to a better understanding of the functional relevance of EHD1 in human tissues, particularly in the kidney and inner ear.
A founder mutation in EHD1 presents with tubular proteinuria and deafness

Running title: Proteinuria, deafness and EHD1

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Abstract

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Conclusion: A previously unrecognized autosomal recessive disorder characterized by sensorineural deafness and tubular proteinuria is caused by a homozygous missense variant in EHD1. Recessive EHD1 variants should be considered in individuals with hearing impairment, especially if tubular proteinuria is noted.
Introduction

Endocytosis refers to the mechanism by which cells internalize macromolecules and particles into transport vesicles derived from the plasma membrane. It is a crucial and regulated pathway for entry into the cell involved in numerous processes, including neurotransmission, signal transduction, immune response and cellular homeostasis. In the kidney, endocytosis is critical for the reabsorption of filtered macromolecules, such as LMW proteins. Investigations of rare diseases associated with LMW proteinuria have identified important roles for several cellular proteins involved in this process. Most filtered macromolecules are retrieved from the proximal tubular lumen by the promiscuous receptors Megalin, Cubilin and Amnionless. Mutations in the encoding genes LRP2, CUBN and AMN cause Donnai-Barrow syndrome (MIM222448) and Imerslund-Grasbeck syndrome (MIM261100 and 618882), respectively. Following endocytosis and release of cargo in the endosome, the receptors are recycled to the plasma membrane, while the cargo is transported to its downstream destination, either the lysosome (degradation) or the basolateral membrane (transepithelial transport). An important regulator of this sorting process is the phosphatidylinositol 5′-phosphatase OCRL, mutations in which cause Lowe syndrome (MIM309000), whereas the chloride/proton antiporter CLCN5 is involved in endosomal acidification and constitutes the molecular basis of Dent disease (MIM300009); both clinically showing LMW proteinuria.

Here we report on our investigations related to patients who presented with LMW proteinuria and sensorineural deafness with neither pathogenic variants in known disease genes nor other defining phenotypes associated with these known disorders. Instead, genetic analysis revealed a homozygous missense mutation in the EHD1 gene.

EHD1 is one of four mammalian dynamin-like C-terminal Eps15 Homology Domain (EHD) proteins and localized to several cytoplasmic vesicular structures, including endocytic vesicles and the Golgi apparatus. The EHD proteins have been previously implicated in endosomal scission, so that receptor
and cargo can be separated in order to be processed to their respective proper destinations. Yet, studies in knockout mice have yielded variable results, ranging from a subclinical phenotype to abnormal sperm development to eye abnormalities to impaired ciliogenesis to embryonic lethality. Interestingly, none of these studies found a role for EHD1 in the kidney or inner ear.
Methods

Full details of all methods can be found in the Supplement.

Ethics

The study was performed in accordance with the Declaration of Helsinki. It was approved by the IRB of the Galilee Medical Center in Naharia (study # 06022007), and by the supreme Helsinki committee of the Israeli Ministry of Health (study # 920070611). The first patient was recruited on May 1st 2008, the last patient was recruited on July 11th 2018. Informed consent was obtained directly from the adult participant and from the parents of participants aged 18 years and younger. Druze ethnicity was self-reported by participants and is reported because of its potential impact on the frequency of genetic variants. All clinical examinations and investigations were performed at the discretion of the treating clinician as part of the patients’ diagnosis and treatment.

Genetic studies

Genotyping, linkage studies and whole exome sequencing were performed as described previously. Variants were assessed using a custom-built in-house software pipeline, as well as the Ingenuity platform (https://variants.ingenuity.com/qci/).

Animal models

Experiments were performed according to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health and were approved by the local councils for animal care. Ehd1 knockout mice were generated by the Sanger Institute, as described previously and acquired from the European Mouse Mutant Archive (MGI ID: 4432418). Ehd1 knockin mice were generated at the Wellcome Trust Centre for Human Genetics, University of Oxford, United Kingdom. Animal experiments on mice to assess renal function were approved by the "Regierung Unterfranken"
Germany. Zebrafish were studied for renal tubular LMW dextran handling as described previously. For details please see the Supplement.

Renal elimination of labelled β₂-microglobulin

A recombinant human β₂-microglobulin expressed in E. coli (Merck, #475828) was conjugated with the fluorescent tag Alexa Fluor™️ 546 and injected into anesthetized mice. After 30 min, urine and blood were collected and tissue fixation was performed. The fluorescence of β₂-microglobulin-Alexa Fluor 546 in urine, serum and kidney lysate samples was measured.

Intravital imaging of proximal tubular protein reabsorption

Mice were anesthetized and the left kidney was exposed. To label the vasculature, a 25 mg/ml solution of FITC-500 kDa Dextran conjugate was used. After 1 min β₂-microglobulin-Alexa Fluor 546 was injected intravenously and the proximal tubular uptake was measured as increase in tubular fluorescence intensity (up to 30 min after injection).

Auditory brainstem response measurements

Anesthetized mice aged 6-9 weeks were used for the measurement of the auditory brainstem response. Stimuli presented were pure tones at 4, 8, 16 and 32 kHz.

Statistics

Data are shown as mean values ± standard error of the mean (SEM); “n” stands for the number of observations. Two-sided unpaired Student’s t-test and ANOVA were used to calculate significance between different groups as appropriate. A p-value ≤ 0.05 was accepted to indicate statistical significance.
Results

Patients

We identified six individuals from four families with an unexplained unique phenotype of LMW proteinuria and sensorineural hearing loss. Proteinuria was discovered incidentally by dipstick (trace to 2+) during investigations for minor illnesses. Four individuals had kidney biopsies performed due to proteinuria, which all were reported as being normal (Figure 1). Renal ultrasound studies showed normal findings. Proteinuria was noted to contain highly elevated levels of LMW protein, including \( \beta_2 \) microglobulin and retinol binding protein, consistent with a defect in proximal tubular protein re-uptake (Figure 1 and Supplemental Table 1).

One individual had a DMSA scan because of suspected urinary tract infection which surprisingly showed globally impaired uptake of the tracer by the kidneys (Figure 1). Subsequently, other affected individuals also underwent DMSA scans with similarly impaired uptake.

The hearing problem was identified during the clinical work-up for this disorder. Audiograms in affected individuals revealed high-frequency hearing loss, consistent with sensorineural hearing impairment (Figure 1).

All affected individuals underwent detailed clinical examinations including formal neurological and ophthalmological assessments and no other abnormalities were identified. No dysmorphologies were noted. Blood studies for renal glomerular function including Vitamin B12 showed no abnormalities. One individual became pregnant and delivered a healthy child. No affected male has so far had progeny. All individuals belong to a Druze ethnic-religious group in Palestine (pedigrees Figure 2).

Genetic studies

Linkage analysis identified a single significant region of interest comprising approximately 1.5 million bases on chromosome 11, with a significant LOD score of 7.2 (Figure 2). This small 1.5 cM locus was defined by two flanking SNPs: rs7131675 and rs2845570. Exome sequencing revealed in the linked
interval a single homozygous variant that segregated with the phenotype, located in EHD1: c.1192C>T; p.(R398W). The damage prediction algorithms indicated functional impairment: CADD score: 32 (likely deleterious); SIFT: 0.01 (deleterious); Polyphen: 0.677 (possibly damaging). This variant is also annotated as rs151119199 in dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and has an allele frequency of 0.00001427 in the gnomAD database (https://gnomad.broadinstitute.org, accessed June 2021). Sequencing of genetically matched healthy Druze individuals identified this variant in one of 196 alleles indicating a strongly increased allele frequency in this population.

**EHD1 and kidney**

In human kidney, we identified EHD1 predominantly in the subapical compartment of proximal tubular epithelial cells (Figure 3 and Supplemental Figure 1). This was confirmed in mouse kidney, where Ehd1 partially co-localized with the endocytic tracer β2-microglobulin and the apical receptor proteins Megalin and Cubilin (Figure 4 and Supplemental Figures 2 and 3). Interestingly, localization and abundance of Megalin and Cubilin appeared unaffected by Ehd1 knockout and knockin (Figure 4 and Supplemental Figure 6). *Ehd1* knockout and antibody specificity were confirmed by the presence or absence of Ehd1 staining in wildtype and *Ehd1* /- mice, respectively (Supplemental Figure 2).

For functional assessment we first assessed *ehd1* in zebrafish. There are two orthologues in zebrafish *ehd1a* and *ehd1b*, both of which are expressed in kidney. When suppressing both paralogues with morpholinos, morphant zebrafish larvae had a significantly impaired ability to reabsorb low-molecular weight dextran compared to control larvae (Supplemental Figure 4).

For a more detailed assessment, we investigated *Ehd1* knockout mice (*Ehd1* /-). We first measured LMW protein uptake using fluorescently labelled β2-microglobulin. This showed a substantial decrease in re-uptake in *Ehd1* /- versus wildtype mice resulting in increased urinary excretion (Figure 3). For better assessment of the R398W variant identified in our patients, we also generated R398W knockin mice (*Ehd1* R398W/R398W). Mutant Ehd1 protein appeared to have largely decreased expression in
proximal tubules and was predominantly localized in small intracellular aggregates (Figure 4, Supplemental Figure 5). In distal nephron segments, mutant Ehd1 was also present in elongated structures that were negative for acetylated tubulin, a marker for cilia (Supplemental Figure 5). These elongated apical structures were not found in wildtype kidneys. In order to gain more mechanistic insights, a possible effect of Ehd1 inactivation on Arf6 and Rab11, two factors involved in trafficking of membranes, was examined. Interestingly, localization and abundance of Arf6 and Rab11 appeared normal in proximal tubules of knockout and knockin mice (Supplemental Figures 6 and 7). Consistent with our patients and \textit{Ehd1}^{-/-} mice, \textit{Ehd1}^{R398W/R398W} mice had increased levels of fluorescent \(\beta_2\)-microglobulin in the urine (Figure 3). Moreover, whereas in wildtype mice reabsorbed fluorescent \(\beta_2\)-microglobulin was mainly confined to the early segments of proximal tubule, in the genetically modified mice it was present throughout the proximal tubule, consistent with compensatory uptake in later segments of the proximal tubule (Figure 3). Similarly, \textit{in vivo} imaging of mouse kidneys using multiphoton microscopy showed a significantly lower \(\beta_2\)-microglobulin uptake rate in \textit{Ehd1}^{-/-} mice (Figure 3 and Supplemental Figure 8).

**EHD1 and inner ear**

Because of the sensorineural deafness of our patients, we investigated expression of Ehd1 in mouse inner ear and identified strong expression in the stria vascularis (Figure 5). Knockout and knockin mice had a loss and altered pattern of Ehd1 expression, respectively (Figure 5). Importantly, \textit{Ehd1}^{R398W/R398W} mice also displayed a significant high-frequency hearing impairment of 20-30 dB compared to age-matched wildtype mice (Figure 5).

**Cellular studies**

We further investigated the cellular consequences of the R398W mutant EHD1 in a proximal tubular cell line, LLC-PK1, genetically modified to either express wildtype or mutant EHD1 when induced with
tetracycline. Cells with wildtype EHD1 showed a spotted distribution pattern as described \(^\text{19}\). In contrast, mutated EHD1 expressing cells showed elongated tubular structures, presumably tubular recycling endosomes, indicating an impairment of membrane fission events (Figure 6). EHD1 is a component of a complex multi-protein membrane shaping machinery. Previous work has indicated that EHD1 together with other proteins, e.g. Pacsin 2, MICAL-L1, and ARF6, functions in endosomal recycling \(^\text{19-21}\). Therefore, we investigated the effect of the EHD1\(^{R398W}\) mutation on the distribution of MICAL-L1 and Pacsin 2. Interestingly, MICAL-L1 remained associated to EHD1 in cells overexpression mutant EHD1 and similarly Pacsin 2. These data indicate that the elongated structures observed in cells with mutant EHD1\(^{R398W}\) are in fact abnormal tubular recycling endosomes (Supplemental Figures 9 and 10).

We also investigated protein stability. After removal of tetracycline, levels of both wildtype and mutant EHD1 were similar, but levels of mutant EHD1 reduced significantly faster, consistent with impaired protein stability of mutant EHD1 (Figure 6). This finding is in agreement with the reduced protein abundance observed in kidneys of \textit{Ehd1} knockin mice (Supplemental Figures 2 and 5).

**Role of \textit{Ehd1} in renal ciliogenesis**

Due to the previously reported role of EHD1 in cilia formation, we assessed cilia morphology \(^\text{14, 22, 23}\). Interestingly, primary cilia were present and appeared to have normal morphology within the proximal tubule in affected individuals, knockout as well as knockin animals (Supplemental Figure 11) \(^\text{24}\).

**\textit{In silico} structural modelling**

Structural analysis identified the mutated EHD1 R398 as part of the \(\alpha\)-helix 12, presumably involved in oligomerization of EHD1 (Figure 7 and Supplemental Figures 12-14). Replacement of arginine with tryptophan at this position is predicted to disrupt the stability of interactions between EHD1 dimers.
leading to mechanically instable oligomerization and the inability to process membrane scission.

Another consequence of the R398W mutation is its possible inference with the nucleotide-binding pocket of an adjacent EHD1 (Figure 7).
Discussion

Our work describes a previously unrecognized syndrome presenting with LMW proteinuria and sensorineural deafness. An isolated population provided us with the opportunity to investigate this EHD1 related syndrome. The unambiguous identification of an identical homozygous genetic variant in four families from the same ethnic background strongly suggests the presence of a founder mutation, consistent with the apparent increased allele frequency of this EHD1 missense variant in the Druze population.

Our clinical, genetic and molecular findings are fully compatible with partial or organ specific loss of EHD1 function as disease mechanism, consistent with autosomal recessive inheritance. The gnomAD database shows that EHD1 is highly constrained and that predicted loss of function variants are significantly less observed than expected (probability of loss of function intolerance = 0.98) suggesting that this ubiquitously expressed protein has a unique and critical biological function. Arguably, complete loss of function may not be compatible with human life and the R398W variant potentially retains some functionality or heterozygous loss of function entails a reproductive disadvantage.

We provide compelling experimental evidence for the role of EHD1 in biology by unequivocally linking it to a distinct human phenotype, i.e. to disease mechanisms related to renal proximal tubular endocytosis and sensorineural deafness. We demonstrate EDH1 related kidney phenotypes in man, in zebrafish as well as in knockout and knockin mice. Impaired renal handling of LMW molecules is seen in our patients, in zebrafish as well as in mice with Ehd1 dysfunction. Remarkably, the renal phenotype was milder in mice than in humans, and decreased absorptive capacity was evident only by administration of labeled β2-microglobulin. Furthermore, we provide evidence for EDH1 related hearing deficits in man and mouse and we speculate that EHD1 dysfunction may interfere with male
fertility based on corroborating data from Ehd1 knockout and additional data from knockin mice (Supplemental Table 2).

Our work identifies EHD1 as a critical component in the endocytic machinery of the renal proximal tubule, aligned with previous work which established a role for EHD1 in endocytic scission and recycling\(^9\). We noted elongated pathological tubular structures in LLC-PK1 cells expressing mutant EHD1 providing yet another piece of circumstantial evidence for EHD1’s role in membrane shaping and fission (Figure 5). In the proximal tubule such scission divides and separates recycling tubules from sorting endosomes, so that receptor proteins handling cargo proteins, such as Megalin can be recycled back to the apical membrane, whereas the cargo proteins can be directed to their respective targets\(^27\). We propose that these EHD1 related mechanisms explain the reduced capacity of proximal tubular endocytosis and subsequent “overflow” of LMW proteins into the urine. Results from our study are fully consistent with this particular role for EHD1 (Figure 7). Interestingly, localization and abundance of Megalin, Cubilin, Arf6 and Rab11 appeared unaffected in Ehd1 knockout and knockin mice (Figure 4 and Supplemental Figures 6 and 7). These data suggest that Ehd1 does not physically bind to these proteins, but that its inactivation or mutation slows down fission of recycling tubules and thus transport rates.

Our data and damage prediction algorithms, such as a CADD score of 32, indicated functional impairment of the p.R398W mutant. A closer look into publicly available structure biology databases revealed how the identified missense mutation p.R398W may impair EHD1 function. On the molecular level, \textit{in silico} modelling suggested that the mutation hinders the formation of EHD1 oligomers necessary for association with endosomes and membrane scission (Figure 7)\(^28\-30\). Thus, structural modeling supported a direct effect on scission and as consequence impaired processing of membranes, receptors and cargo. Moreover, the identified \textit{EHD1} mutation can also interfere with the
nucleotide-binding site of an adjacent EHD1 (Figure 7). This particular mechanism was further supported by the extensive tubulation observed in renal proximal tubular cells expressing mutant EHD1 (Figure 6), which in fact is reminiscent of the cellular phenotype of ATPase-deficient mutant T94A in Ehd2. However, our data do not allow to specify the extent of loss of function for the disease causing R398W variant, yet, the similarity in the phenotypes of Ehd1−/− and Ehd1R398W/R398W mice strongly suggests that it indeed significantly impairs organ specific functions.

A search of the Shared Harvard Inner-Ear Laboratory Database (SHIELD) revealed that Ehd1 is known to be expressed in the mouse inner ear. The association of EHD1 dysfunction with sensorineural deafness and its expression in stria vascularis (Figure 5) highlights the important role of receptor-mediated endocytosis in inner ear function, consistent with the deafness seen in Donnai-Barrow syndrome, due to mutations in Megalin. While the exact role of Megalin in inner ear function is still unclear, it has been implicated in endocytosis in the endolymphatic sac with prominent expression in vestibular dark cells as well as stria vascularis.

Male Ehd1 knockout mice have previously been shown to be infertile. Our results corroborate this and, importantly, male Ehd1R398W/R398W mice are also infertile (Supplemental Table 2). However, if this also applies to humans remains to be seen as the young ages of our male patients do not allow any conclusions.

Of further interest is our finding of morphologically normal primary cilia in both patients and genetically modified mice (Supplemental Figure 11) as this is in contrast to previous reports of defects in ciliogenesis in zebrafish and Ehd1 knockout mice. Ciliopathies typically present in the kidney with cysts, which were not seen in this study. Our findings argue against an essential role of EHD1 in
ciliogenesis, at least in the kidney. Of note, defects in ciliogenesis in mice appeared to be dependent on the genetic background of mice \(^{13, 14}\).

In summary, a previously unrecognized syndrome, characterized by LMW proteinuria and sensorineural deafness accompanied by markedly reduced DMSA uptake in renal imaging, is caused by mutated \textit{EHD1}. Our findings enable an accurate diagnosis, genetic testing and counselling for affected individuals and families. Our findings are consistent with a role for \textit{EHD1} as an intracellular membrane-shaping protein involved in vesicular trafficking and recycling. Our work also establishes \textit{EHD1} as a new deafness disease gene. Because of the subtlety of the kidney phenotype, which may go unrecognized in clinical practice, \textit{EHD1} variants should be considered in patients with apparent isolated deafness. Moreover, our study suggests a possible role for \textit{EHD1} in male infertility, thereby corroborating a previous study \(^{10}\).

The identification of mutated \textit{EHD1} being causative for a rare disease entity in an isolated population with its well-defined phenotypes provides solid evidence for \textit{EHD1}'s real role in life. These insights position \textit{EHD1} a) as reasonable biological target for the prevention of treatment (e.g. cancer drugs) related renal toxicity linked to proximal tubular transport processes \(^{35}\), b) as protein of interest for an improved understanding of the biology of inner ear function and hearing problems, and potentially also c) for a role in male fertility.

**Author Contributions**

All authors together generated and gathered the patient, animal, genetic and molecular data and analyzed the data. Drs. Robert Kleta, Tzipora C. Falik Zaccai, and Richard Warth vouch for the data and the analysis. All authors helped writing the paper, and all together decided to publish this paper.
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We are grateful to all of our patients and their families for their kind and significant engagement.

Disclosures

D. Magen reports Consultancy: Alnylam Pharmaceuticals; Research Funding: Alnylam Pharmaceuticals; and Honoraria: Alnylam Pharmaceuticals. B. Davies reports Advisory or Leadership Role: International Society of Transgenic Technologies. R. Warth reports Advisory or Leadership Role: Editorial Board of JASN and Pflügers Archive; and Other Interests or Relationships: Member of the German Society of Nephrology and German Physiological Society. P. Oefner reports Ownership Interest: PDL BioPharma Inc.; and Advisory or Leadership Role: Editorial Board of BioTechniques, Communicating Editor of Human Mutation, Editorial Board of BioMed Research International, Oncology Section, and Editorial Board of Metabolites. E. Klootwijk reports Patents or Royalties: New Zealand Pharmaceuticals. All remaining authors have nothing to disclose.

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**Figures legends**

**Figure 1: Human phenotype**

Shown are key phenotypical features noted in affected individuals. (A) Bar graphs illustrating proteinuria with horizontal dashed black lines indicating the upper limits of normal. 24-h urine protein excretion (left bar) ranges from 0.67 to 2.1 g/d. Proteinuria was predominantly low molecular weight proteinuria as indicated by the highly elevated levels of $\beta_2$-microglobulin (middle bar) and retinol binding protein (RBP, normalized to urinary creatinine, right bar). (B) Affected individuals had impaired renal uptake of DMSA. Shown are representative single photon emission tomography (SPECT) images from a healthy control (left panel) and individual 1.1 (right panel) 4 hours after injection of DMSA. Note the markedly decreased global uptake in the affected individual. The bar chart summarizes the results from individuals 1.1, 2.3, 3.1, 3.2, and 4.1.; normal uptake is indicated by the horizontal dashed black line. (C) Patients had normal kidney histology. Shown is a representative image from a kidney biopsy of individual 1.1 stained with hematoxylin and eosin (H&E). Note the normal glomerular, tubular and interstitial morphology (scale bar 50 µm). (D) Affected individuals had sensorineural deafness. Shown is a representative audiogram from affected individual 4.1 (solid line). Note the pronounced hearing loss for higher frequencies compared to control (dashed line).

**Figure 2: Genetics**

(A) Shown are the pedigrees of four families studied suggesting autosomal recessive inheritance. Females are represented by circles and males by squares. A double line between parents indicates consanguinity. Affected individuals are denoted by filled symbols. The numbers below these symbols denote individuals as referred to in the text. (B) The combined multipoint parametric linkage analysis shows a single genome wide significant peak on chromosome 11, with a maximum LOD score (y-axis) of 7.2. Genetic distance (in centimorgan) and individual chromosomes (1 to 22) are indicated on the lower and upper X-axes, respectively. (C) Representative sequence chromatograms. The EHD1 variant c.1192C>T (indicated by an arrow) is homozygous in an affected individual (left upper panel), absent in the reference sequence (right upper panel) and heterozygous in both parents (lower panels). (D) Regional homology plot of the protein sequence of EHD1 around the change of amino acid 398 from arginine to tryptophan (indicated by arrow). Note the strict and complete evolutionary conservation of R398.
Figure 3: EHD1 localization and function in the kidney

(A) Localization of EHD1 (green) in the normal human proximal tubule. Please note strong EHD1 signal in the subapical compartment beneath the tubular lumen. Nuclear staining (blue); scale bar 10 µm.

(B) Normal human kidney showing very little or absent expression of EHD1 (green) within the glomerulus and EHD1 prominence in adjacent proximal tubules. Nuclear staining (blue); scale bar 50 µm. (C-E), Kidney sections showing reabsorption of fluorescently labeled β2-microglobulin (white) (30 min after injection) in proximal tubules in a wildtype (C), knockout (D) and knockin mouse (E). Note, in wildtype mouse kidney (c) reabsorption of fluorescently labeled β2-microglobulin was observed mainly in early proximal tubules (S1 and S2 segments). In kidneys of homozygous Ehd1 knockout (D) or Ehd1R398W/R398W mice (E), reabsorption of β2-microglobulin was not complete after passage of tubular fluid through the early portions of the proximal tubule. Therefore, reabsorption was also observed in late proximal tubules and a spillover of β2-microglobulin into urine (F) was observed. Scale bars 100 µm. (F) Summary of urinary excretion of labeled β2-microglobulin during 30 min. Please note the increased urinary loss in homozygous Ehd1 knockout (KO) and knockin (R398W) mice. Asterisks indicate p ≤ 0.05 (ANOVA with Dunnett’s multiple comparison test). (G) Summary of intravital multiphoton microscopy revealing decreased reabsorption of fluorescently labeled β2-microglobulin (10 min after injection) into proximal tubules of Ehd1 knockout mice (symbols indicate individual tubules, 6 animals each group). Asterisk indicates p ≤ 0.05 (t-test).

Figure 4: EHD1 localization in relation to Cubilin and Megalin

In wildtype mice (upper row), Ehd1 (green) was predominantly localized in the subapical compartment. Cubilin is shown in red, Megalin in blue, cell nuclei (DAPI) in magenta. In knockout mice (middle row) and knockin mice (lower row), the localization of Cubilin and Megalin appeared unaffected. In knockin mice, Ehd1 abundance was reduced and mutant Ehd1 was observed in intracellular tubules. Left four panels are confocal images; right high magnification panel: STED image for Ehd1, Cubilin, and Megalin. Deconvolution of all images was performed using Huygens software. Scale bar: 5 µm.
Figure 5: Ehd1 and inner ear

(A) Localization of Ehd1 (green) in stria vascularis (inner ear). In wildtype mice, Ehd1 was strongly expressed in the stria vascularis (SV). In homozygous knockout (B) and knockin mice (C) the labeling of stria vascularis (SV) and Reissner membrane (RM) was absent or grossly diminished, respectively. (SM) scala media (containing endolymph); scale bar 50 µm. (D) Auditory brainstem response measurements of mice revealed a high-frequency hearing impairment in homozygous knockin mice (Ehd1^R398W/R398W, n=6, red symbols) compared to wildtype mice (Ehd1^wt/wt, n=7, black symbols). Please note the reverse scaling of the y-axis to facilitate comparison with the hearing phenotype of the patient shown in Figure 1. Asterisks indicates p ≤ 0.05 between groups (t-test with Bonferroni-Dunn correction for multiple testing).

Figure 6: Cellular consequences of mutant EHD1 and protein stability

(A) Immunostained human EHD1 in inducible porcine proximal tubular cells (LLC-PK1). Please note the “spotty” pattern in cells expressing wildtype EHD1 (left panel) and long intracellular structures, presumably tubular recycling endosomes, decorated with EHD1^R398W. (B) Western blot of cell lysates after removal of tetracycline that was used to induce expression of wildtype or mutant EHD1. Please note the faster decay of EHD1 protein in cells expressing mutant EHD1. (C) Summary of experiments as depicted in (B). EHD1-WT (white bars, filled black symbols), EHD1^R398 (gray bars, open symbols). Error bars indicate SEM, asterisks p ≤ 0.05.

Figure 7: Structural consequences and cell model

(A) Three copies of the activated EHD1 homology model were arranged as oligomer similar to the packing of EHD4 in the crystal (pdb entry code 4CDI). A potential arrangement of the EHD1 oligomer (here trimer of dimers) at the membrane is visualized. The KPF loop (colored in yellow) of one dimer facilitates major dimer-dimer contacts allowing for a repetitive back-to-front arrangement of EHD1 dimers. (B) Homology modelling suggested that Arg398 located at the tip of α12 faces towards the dimer-interface. A possible interaction that would contribute to the dimer-interactions is Glu106-Arg398, which would further stabilize the interaction of the KPF loop (Pro110-Arg135) with α12. (C) Side-on view of oligomeric form of EHD4. Membrane binding is predicted to be at the bottom. The arrow indicates the position of the mutated residue at equivalent position 398 in EHD1. (D) Zoomed
view of the tryptophan (W) at the equivalent position 398 in human EHD1. The W is shown in stick representation, projecting close to a loop of the nucleotide-binding pocket. ATPγS is shown bound in this pocket in space filling representation. Note the close proximity of the W residue, which is likely to constrain nucleotide binding, hydrolysis or release by EHD1.

(E) Simplified model of endocytosis and recycling of membranes and receptors in normal proximal tubules. (1) At the base of the brush border membrane, after Megalin and Cubilin (not shown) bind their ligands, such as filtered proteins, the plasma membrane forms invaginations, mostly via Clathrin-coated pits, which lead to the formation of endosomes (2). The endosomes are further processed into sorting endosomes (3) and the endocytic recycling compartment (SE/ERC). Low pH in vacuoles contributes to the separation of ligands from their receptors (4). From the endocytic vacuoles/recycling endosomes, these dissociated ligands are directed to multivesicular bodies (not shown) and finally to lysosomes. Receptor-containing vesicles are redirected to the apical membrane (via dense apical tubules). EHD1 and its dimers/oligomers are involved in fission of the endocytic tubules (5) and support receptor recycling (6). (F) Tubule fission is impaired in EHD1 patients and knockin mice. Mutant EHD1 dysfunction slows the recycling rate and leads to renal tubular proteinuria, as demonstrated in affected individuals and in animal models. In addition, mutant EHD1 forms aggregates in the cytosol. Megalin and Cubilin are not found in these aggregates, suggesting that EHD1 does not physically interact with these receptor proteins.
Figure 1: Human phenotype

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153x168mm (300 x 300 DPI)
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Supplement

A founder mutation in EHD1 presents with tubular proteinuria and deafness

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Supplemental Methods

**Ehd1 knockout mice**

Experiments were performed according to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health and were approved by the local councils for animal care according to the German law for animal care. Animal experiments on mice to assess renal function were approved by the Regierung Unterfranken, Germany.

We thank the Wellcome Trust Sanger Institute Mouse Genetics Project (Sanger MGP) and its funders for providing the mutant mouse line C57BL/6NTac-Ehd1tm1a(EUCOMM)Wtsi/WtsiBiat (https://www.mousephenotype.org/data/alleles/MGI:1341878/tm1a(EUCOMM)Wtsi), and the European Mouse Mutant Archive (https://www.infrafrontier.eu; EMMA ID: EM:05712) from which the mouse line was received. These mice carrying a floxed exon 2 of Ehd1 were bred with a global 129/Sv Cre Recombinase pCX-NLS Cre mouse. In the offspring deletion of exon 2 was confirmed and mice were crossed to obtain homozygous progeny for the Ehd1 gene deletion (Ehd1–/–).

**Ehd1R398W/R398W knockin mice**

The Ehd1 knockin mice were generated in house (Wellcome Centre for Human Genetics, University of Oxford, United Kingdom) using Crispr/Cas9 technologies. A CRISPR/Cas9 nuclease was designed against the sequence 5'-GATGGTGATGGTGCGCCAGG-'3 within exon 5 (ENSMUSE00000232103) of the mouse Ehd1 gene, the target site of which encompasses the Arginine-398 residue which we identified to be mutated to a Tryptophan residue in families with hereditary tubular proteinuria and hearing deficit. A single stranded oligonucleotide harboring the desired point mutation was designed and used as a template for homology directed repair to introduce the orthologous mutation into the mouse genome. The single-strand oligodeoxynucleotide (ssODN) and the guide-RNA, prepared by in vitro transcription, for the designed CRISPR/Cas9 nuclease were microinjected into fertilized C57BL/6J oocytes prepared from transgenic female mice that overexpress Cas9. In the resulting litters, multiple founder mice were generated which harbored the desired R398W mutation. The production of the knockin mice was carried out in accordance with UK Home Office Animal [Scientific Procedures] Act 1986, with procedures reviewed by the Clinical Medicine Animal Welfare and Ethical Review Board at the University of Oxford, and conducted under project license PPL 30/3085.

**Immunostaining**

Mice were sacrificed by exsanguination in deep anesthesia (2.5% isoflurane). Immediately post mortem, the animals were perfused via the abdominal aorta with 3% paraformaldehyde dissolved in a solution of 100 mM sucrose, 90 mM NaCl, 15 mM K2HPO4, 1 mM EGTA and 2 mM MgCl2 (pH 7.4). The fixed kidneys were removed and either frozen in liquid nitrogen for cryo-sectioning or further processed for standard paraffin embedding.

For immunofluorescence staining on paraffin-embedded tissue, the sections were deparaffinized and epitope unmasking was performed by incubation in citrate buffer (pH 6.0) at 95°C for 15 min. On cryo-sections epitope unmasking was performed by incubation in 0.1% SDS solution for 5 min. Unspecific
antibody binding sites were blocked using 5% BSA solution for 10 min. Primary and secondary antibodies were diluted in PBS based solution containing 0.5% BSA and 0.04% Triton X-100. Primary antibody incubation was performed overnight at 4°C. Tissue sections were washed with PBS before adding the secondary antibody for 1 h at room temperature. Sections were mounted using DAKO glycergel mounting medium (Agilent). Immunofluorescence signals of stained sections were analyzed using an inverted microscope (Axiovert 200, Zeiss) or a confocal microscope (LSM 710, Zeiss).

Stimulated-Emission–Depletion (STED) super-resolution microscopy

For STED microscopy, Cryosections for Rab11A staining were antigen retrieved using 0.5% tritonX-100 in PBS. Paraffin sections were used for triple-antibody staining of EHD1/Cubilin/Megalin. Heat-induced antigen retrieval were performed in citrate buffer (pH 6). Sections were blocked with 10% donkey serum/PBS and sequentially incubated with primary antibodies in 5% donkey serum/PBS overnight. Suitable StarRed-, Star580-, Alexa488-coupled secondary antibodies (Abberior, Göttingen, Germany; Dianova, Hamburg, Germany) were used. Counter staining were performed using Acti-stain 488 Phalloidin (Cytoskeleton) and/or 4',6-diamidino-2-phenylindole. Sections were mounted with Abberior Liquid Mount and analyzed using a multilaser confocal scanning microscope and/or stimulated-emission-depletion super-resolution microscopy (see below).

Images were acquired using Facility Line (Abberior Instruments, Göttingen, Germany) with Olympus IX83 microscope (Germany) and Imspector software (Abberior Instruments) in confocal microscopy mode or STED mode. The images were de-convolved using Huygens Professional Software (version 20.10, Scientific Volume Imaging B.V., Netherlands). The Classic Maximum Likelihood Estimation (CMLE) algorithm for deconvolution was performed using standard setting (quality change threshold 0.1%).

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Fluorescent labeling of β2-microglobulin

1 mg protein of recombinant, human β2-microglobulin expressed in E. coli (Merck) was used for conjugation with the fluorescent tag Alexa Fluor™ 546 using the Alexa Fluor™ 546 Protein Labelling Kit (Thermo Fisher) according to the manufacturer’s instructions. The conjugates were separated from the unconjugated dye by exclusion purification resin. The obtained β2-microglobulin-Alexa Fluor 546 conjugate had a concentration of ~750 µM.

Reabsorption of β2-microglobulin

To assess renal reabsorption of β2-microglobulin, age-matched mice were anesthetized using isoflurane inhalation (1.5% isoflurane in a gas mixture of 50% oxygen and 50% nitrogen). A catheter was placed into the femoral vein, the bladder was emptied and a 1:50 dilution (in 0.9% NaCl) of the above-mentioned Alexa Fluor 546 labeled β2-microglobulin was injected into the femoral vein at a dose of 20 µl/g body weight. 30 min later, urine and blood were collected and the mice were sacrificed by exsanguination via the vena cava. Immediately post mortem, animals were perfused with fixative via the abdominal aorta. Both kidneys were harvested; one was homogenized in distilled water, the other one was prepared for cryo-sectioning. The fluorescence intensity of Alexa Fluor 546 in plasma, urine and kidney homogenate was measured using a microplate reader (NOVOstar microplate reader).

RNAscope of kidneys

RNAscope experiments were performed on paraffin-embedded kidneys using the RNAscope® 2.5 HD Assay Kit (ACD) according to the manufacturer’s instructions.
Auditory brainstem response measurement

Animals aged between 6-8 weeks were used for the measurement of the auditory brainstem response. Mice were anaesthetized using a mixture of ketamine (96 mg/kg), xylazine (4 mg/kg) and physiological NaCl solution at a mixing ratio of 9:1:8, initial dose: 0.3 mL s.c.. Mice were placed within a sound-attenuated chamber on a thermally controlled heating pad at 37°C, and frequency-specific auditory brainstem responses (f-ABR) were measured. The protocol was performed as previously described 4. Briefly, f-ABR were measured via subcutaneously placed thin silver wire electrodes (0.25 mm diameter) using a low noise amplifier (JHM NeuroAmp 401, J. Helbig Messtechnik, Mainaschaff, Germany; amplification 10,000; bandpass filter 400 to 2,000 Hz and 50 Hz notch filter) in combination with a custom-made Python program (Python 2.6) for stimulation and data recording. Auditory stimuli of 4, 8, 16 and 32 kHz were presented free-field to one ear at 3 cm distance from the animal's pinna via a custom-made speaker. The speaker’s frequency response function was corrected to be flat within ±1 dB. Stimuli presented were pure tones (6 ms duration including 2 ms cosine-squared rise and fall times) between the range of 1 and 32 kHz. 300 stimuli were presented with alternating inverted phase with a repetition rate of 4 Hz. The stimuli of all tested frequencies were presented pseudorandomized with different sound pressure levels between 30 dB and 100 dB with a step width of 5 dB. To obtain the f-ABR-based hearing thresholds, the mean sound intensity dependent f-ABR wave root-mean-square values independent for each frequency were fitted by hard-sigmoid functions. Thresholds were defined automatically by a custom-made Python program at the inflection point of that hard sigmoid fit. Variance of the calculated ABR thresholds were obtained by data subsampling. Data were discarded at frequencies where this procedure was not possible, for example, at very low signal-to-noise ratios. The statistical analysis of the individual and group threshold data was performed in Origin.

Intravital microscopy of renal proximal tubular endocytosis

Mice were anesthetized through inhalation of isoflurane (1.5-2.5% isoflurane). The body temperature was maintained at 37°C by placing the animals on an operating table with a servo-controlled heating plate. A cannula connected to a syringe was inserted into the right jugular for the intravenous infusion of the dyes. For kidney imaging, the left kidney was exposed by making a small flank incision. The experiments were performed using a Zeiss LSM 710 confocal fluorescence microscope. Excitation was achieved using a Chameleon Ultra-II MP laser (Coherent Deutschland, Dieburg, Germany) at 940 nm with a laser power of 20% of 3200 mW. Eight-bit 1454 x 1454 pixel images (providing a theoretical dynamic range for intensities measurements of 0–65536 pixel intensity) were obtained using a pixel dwell time of 3.15 μs and a line average of one by applying a 40x long distance (LD) C-Apochromat 40/1.1 water objective. The emissions were collected using external detectors: Nondescanned detectors with filter set 1 (green channel): beam splitter 500–550 and long pass (LP) 555 and filter set 2 (red channel): beam splitter P 565–610 including mirror. The detector settings were kept constant for all measurements: for the green and red channels, respectively, the master gain was 600/600, the digital gain was 10/10, and the offset was -0.0/0.0. To label the vasculature, a 25 mg/ml solution of FITC-500 kDa Dextran conjugate dissolved in PBS was first concentrated using Nanosep Centrifugal (VWR International, Darmstadt, Germany) and injected intravenously (0.37 μl/g body weight). The
fluorescence was detected using the green channel. After 1 min, the Alexa Fluor 546-labeled β2-
microglobulin dissolved in PBS was injected intravenously (1.56 μl/g body weight) and the proximal
tubular β2-microglobulin uptake was measured as the increase in the tubular fluorescence intensity
during the 30 min after injection. The fluorescence was detected using the red channel. The mean
fluorescence intensity of Alexa Fluor 546-labeled β2-microglobulin measured in 3–6 proximal tubules
per animal over time (30 min, 15 images) and the background readings for the proximal tubular
autofluorescence before labeled β2-microglobulin injection were assessed using ImageJ V1.37c. The
reabsorption capacity of the proximal tubules was compared after 2, 4, 10, 20 and 30 min of β2-
microglobulin injection by assessing the mean fluorescence intensity of β2-microglobulin per tubule and
subtracting the background fluorescence.

Zebrafish strains and husbandry
Zebrafish were raised and maintained at the University of Manchester Biological Services Unit
according to the UK Animals Act 1986. The wildtype line was of the AB background.

Zebrafish RNA isolation, RT-PCR and Q-PCR
Total RNA was isolated from zebrafish embryos using Trizol (Invitrogen) and reverse-transcribed with
Superscript First Strand (Invitrogen) to produce cDNA. For direct visualization of amplification products,
cDNA was amplified using standard PCR conditions and appropriate primer pairs. Q-PCR was
performed using SYBR Green (Sigma-Aldrich) according to the manufacturer’s protocol. 0.5 μl cDNA
template from a 20 μl aliquot generated from 5 μg of RNA was used per reaction. Each experiment was
run in duplicate and was repeated on three individually obtained RNA extracts. Studies were performed
using the ABI PRISM 7000 sequence detector system (Applied Biosystems Ltd).

Morpholino injections into zebrafish
Morpholinos were obtained from GeneTools. The standard control morpholino had the sequence
CCTCTTACCTCAGTTACAATTTATA (https://www.gene-tools.com/content/negative-control-
morpholino-oligos). Morpholinos targeting zebrafish ehd1 were the same as described previously, with
the following sequences: ehd1a (CTGAACATGGTGGACGTTACACGAC); ehd1b (ATCTTTGTTAGACCAACTGAACATT) and were injected with morpholino targeting p53 (1 nl of a 250
μM stock) into one cell stage embryos as described previously 5, 6.

Injection and analysis of endocytic tracer
Lysine-fixable 10 kDa dextran labelled with Alexa 488 (Molecular Probes) was prepared in PBS at 2
μg/μl final concentration, and 1 nl was injected into the larval circulatory system. Zebrafish larvae at 4
dpf were anesthetized with 0.2 mg/ml MS222 (Sigma) in chorion water, and tracer injected into the
common cardinal vein using a glass micropipette PLI-90 Pico-Injector (Harvard Apparatus). Pronephric
uptake was assessed at 2.5 h on whole mounts using a fluorescence dissecting stereomicroscope
(Leica MZ10F). Statistical analysis was performed using the Pearson’ s chi-squared test with Prism
software (Prism Software Corporation).
Western blotting of zebrafish larvae

Larvae were culled by anaesthetic overdose and 100 collected per condition in 1.5 mL Eppendorf tubes. Embryos were then triturated ~15 times in Ginzburg fish ringers’ solution (110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl₂·2H₂O, 10 mM Tris pH 8.5) containing 0.75 mM EDTA, 0.3 mM PMSF, and 20x protease inhibitor cocktail to remove the yolk sac. Larvae were centrifuged at 1800 rpm for 1 min, supernatant removed, and washed again in Ginzburg solution before analysis by Western blotting with antibodies to EHD1 (Steve Caplan, University of Nebraska) and GAPDH (Santa Cruz SC-25778).

Ethics statement on Zebrafish experiments

All work was performed under the UK Home Office animal project license number 70/9091. Local animal care was provided by the University of Manchester BSF Unit. Zebrafish larvae at 4 dpf were anesthetized with 0.2 mg/ml MS222 for injection of endocytic tracer. Following the experiment, euthanasia was performed by incubation in 0.2 mg/ml MS222 for >2h.

Proteomics

Mouse kidney was transferred to 2-ml Precellys tubes (Precellys® Keramik-Kit 1.4/2.8 mm, Bertin). Homogenisation was performed twice at 6,500 rpm for 20 sec in 1 ml of ice cold 80% MeOH by means of the Precellys® 24 Homogenisator (Bertin). Samples were then incubated at -20 °C overnight prior to centrifugation at 20,000 x g for 15 min at 4°C. Supernatant was discarded and the pellets were air-dried. Further lysis was performed in 1 ml of gel-aided sample preparation (GASP)-buffer 7 supplemented with 20 mM DTT. Total protein amount was determined using the SERVA Purple Protein Quantification Assay (SERVA Electrophoresis GmbH) according to the manufacturer’s instructions. Fifty micrograms of protein lysate were then processed using the GASP protocol as described previously 7. After tryptic digestion, the peptide extracts were redissolved in 25 µl of 5% formic acid.

Microliquid chromatographic (microLC) separations of tryptic digests were performed on an Eksigent LC 425 (AB Sciex) coupled to an AB Sciex TripleTOF 5600+ mass spectrometer 6. The tryptic digests (5 µg each) were injected directly onto a 150 × 0.3 mm l.d. CSH-C18 column (particle size 1.7 µm, 120 Å, Waters) and peptides were separated at a column temperature of 40 °C using a 85-min linear acetonitrile gradient (3–40%) in 0.1% formic acid atflow-rate of 6 µL/min. Samples were injected once each for mass spectrometric (MS) analysis in data-dependent acquisition (DDA) mode and Sequential Window Acquisition of all THeoretical fragment-ion spectra (SWATH), respectively 9. For peptide library generation, the TripleTOF 5600+ mass spectrometer (AB Sciex, Darmstadt, Germany) was operated in DDA mode from 400–1,000 m/z for 250 ms, followed by acquisition of MS/MS-spectra from 230–1,500 m/z of the 20 most intensive precursor ions for 50 ms per precursor. The data were searched using ProteinPilot 5.0 (AB Sciex) against the UniProtKB/Swiss-Prot (Version 03-2021) database. For SWATH-MS, an initial 50 ms TOF-MS full scan was employed, before the entire m/z range of 230–1,500 was covered using 60 SWATH variable windows of 50 ms each 10. For quantification of the proteins from the SWATH runs the PeakView 2.2 software (AB Sciex) and the MicroApp 2.0.1 were used.
Homology modelling of EHD1 and EHD1^{R398W}

Human EHD isoforms EHD1, EHD2 and EHD4 exhibit high sequence identities of about 70% (Supplemental Figure 7). Two homology models of EHD1 were generated based on crystal structures of the autoinhibited state of EHD2 (pdb entry code 4CDI) (Supplemental Figure 8A and 8B) and the activated state of EHD4 (pdb entry code 5MTV) (Supplemental Figure 8C and 8D) as respective templates. Arg398 in both models is located at the very end of helix α12 in front of the linker loop to the EH-domains pointing away from the dimer interface and the GTPase domain (Supplemental Figure 8B and 8D). At a first glance, a functional role for Arg398 and the phenotype seen in EHD1^{R398W} is not obvious from the homology models. However, looking into the activation mechanisms of EHD proteins points towards a problem in oligomerization as consequence of the R398W mutation found in our patients.

The auto-inhibited state differs from activated state in EHDs in the orientation of helix α8, which rotates over 60° around a hinge point at Pro286 (Supplemental Figure 7, black star and Supplemental Figure 8). In the inhibited state, α8 is wedged between the N-terminal helical domain α(1a,b) and a bundle of three small helices α9 - α11 (pink in Supplemental Figure 8B and 8D) which are known to be involved in membrane interactions. Activation is suggested to start with a conformational change of the N-terminal domain at the membrane surface, thereby releasing α8, which snaps like a Swiss army knife into the extended conformation (Supplemental Figure 8C and 8D). This rigid-body movement is accompanied by several changes such as the detachment of the EH-domain from the GTPase domain, re-arrangement of the membrane interacting bundle α9 - α11 (Supplemental Figure 8B and 8D), and re-folding and relocation of the KPF loop. None of these conformational changes seem to involve Arg398, which rather faces away from the KPF loop within a monomer. However, in the active state EHD1 dimers oligomerize in a front-to-back orientation mediated by the KPF loop of individual dimers (Supplemental Figure 9A and 9B). In the context of this oligomerization Arg398 in EHD1 might provide a re-enforcement of dimer-dimer interaction. A likely partner candidate is Glu106 that is located just in front of the KPF loop of the adjacent dimer. A Glu106-Arg398 interaction (Supplemental Figure 9B) might help sculpturing the oligomerization interface further.

Ab initio mutation against tryptophan in the homology model of EHD1^{R398W} did not alter the overall conformation of the EHD1 dimer but the introduction of a bulky and hydrophobic residue like tryptophan would affect this most relevant oligomerization interface (Supplemental Figure 9C). In addition, Trp398 might form inter-monomer interaction, e.g., with Arg287, linking α8 - α12 together. Any change along the dimer-dimer interface will affect the interaction of the KPF loop with the membrane interacting helices (pink in Supplemental Figure 9C). We suggest that even subtle reorientations of the α9 - α11 bundle with respect to the membrane plane might alter the geometry of membrane fission and could cause the phenotypical change in membrane morphology in EHD1^{R398W}. 
Another hypothesis explaining the functional deficit of the R398W mutant is based on the seminal works of Daumke et al. 11, 12. EHD1 and EHD4 (mouse EHD4 ADP conformation structure: pdb 5MVF; mouse EHD4 ATP conformation structure: pdb 5MTV) are well conserved at the protein level, and the published sequences of EHD2 (mouse EHD2 pdb 2QPT) and EHD4 are very similar, so we are confident the modeling is accurate. According to our modeling, the mutated R398 of human EHD1 lies very close to the nucleotide-binding pocket of an adjacent EHD1 protein within the EHD1 oligomer, based upon the crystal packing structure (Supplemental Figure 10). The conversion of R398 to W would result in a likely physical constraint of the loops constituting the nucleotide-binding pocket. This, we predict, would either interfere with nucleotide binding, nucleotide hydrolysis, or release of nucleotide following catalysis. We favor the last two based upon the cellular phenotype of the mutant, which is membrane-bound (suggesting binding to ATP), and able to drive membrane tubulation consistent with a lack of membrane fission (Supplemental Figure 11). This in fact is similar to the phenotype seen with ATPase-deficient mutant T94A in EHD2 11 strongly arguing for this mechanism as at least a contributing mechanism.

Statistics

Data are shown in mean values ± standard error of the mean (SEM); “n” stands for the number of observations. Unpaired two-sided Student’s t-test and ANOVA with Tukey’s or Dunnett’s multiple comparison tests were used to calculate significance between different groups or time series, respectively. A p-value ≤ 0.05 was accepted to indicate statistical significance, which was identified by an asterisk (*). Statistics were performed using Origin V94E (OriginLab Corporation), SPPSS and GraphPad Prism software.
Supplemental Tables

Supplemental Table 1: Clinical phenotype details

<table>
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<th>Patient</th>
<th>Age at diagnosis [years]</th>
<th>Albumin / creatinine ratio [mg/g]</th>
<th>24-h urine protein [g]</th>
<th>β2M [mg/l]</th>
<th>Plasma Creatinine [mg/dl]</th>
<th>DMSA Renal uptake left / right [%]</th>
<th>Audiogram</th>
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<td>11</td>
<td>189</td>
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<td>121</td>
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<td>10</td>
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<td>1.0</td>
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<td>0.6</td>
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<td>SND</td>
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<td>160</td>
<td>0.8</td>
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<td>SND</td>
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<tr>
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<td>0.7</td>
<td>150</td>
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<td>&lt; 0.15</td>
<td>&lt; 1.8</td>
<td>0.2-1.0*</td>
<td>&gt; 21 / &gt; 21</td>
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Shown are pertinent clinical and biochemical data. Note proteinuria, which is predominantly low-molecular weight, as indicated by the elevated β2-microglobulin (β2M).

Note also the very low uptake of ⁹⁹mTc-dimercaptosuccinic acid (DMSA) by the kidneys in the five tested affected individuals. Renal scintigraphy was performed four hours after administering 0.5mCi/kg of DMSA intravenously. The images were acquired using a double-head scintillation gamma camera equipped with a low-energy-high-resolution collimator (Infinia, General Electric, Haifa, Israel). Posterior planar images in the supine position were acquired with a 256x256 matrix and zoom=1 until 250,000 counts were detected. Single photon emission computed tomography (SPECT) images were acquired with a 64x64 matrix, zoom=1, 120 projections 3° apart with 15 second/projection. Renal uptake was calculated using the method detailed by Groshar et al. (Quantitation of Renal Uptake of Technetium-99m DMSA Using SPECT. J Nucl Med. 1989;30:246-250). Kidney volumes and radioactive concentration measurements were calculated using a customized computer program. For each kidney the injected dose (ID) per cc (%ID/cc) was measured and multiplied by the kidney volume for the calculation of the total kidney uptake. The patient uptake values were compared to uptake values in a normal population.

Moreover, all patients have sensorineural deafness (SND).

*The creatinine reference is age dependent: at the age of the youngest participant (5 years) it would be 0.2-0.5 mg/dl, and of the oldest (a female): 0.7-1.0 mg/dl. Note that plasma creatinine values are in the normal range for all. ND: not done.
**Supplemental Table 2: Breeding statistics**

<table>
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<tr>
<th>n</th>
<th>female</th>
<th>male</th>
<th>$Ehd1^{+/–}$ pups</th>
<th>$Ehd1^{+/–}$ pups</th>
<th>$Ehd1^{+/-}$ pups</th>
<th>$Ehd1^{+/-}$ pups</th>
<th>$Ehd1^{+/-}$ pups</th>
<th>$Ehd1^{+/-}$ pups</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>6*</td>
<td>$Ehd1^{+/-}$</td>
<td>$Ehd1^{+/-}$</td>
<td>60 (28%)</td>
<td>132 (61.7%)</td>
<td>22 (10.3%)</td>
<td>4 (0.0%)</td>
<td>1 (0.0%)</td>
<td>214</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$Ehd1^{+/-}$</td>
<td>$Ehd1^{+/-}$</td>
<td>0</td>
<td>4 (80.0%)</td>
<td>1 (20%)</td>
<td>4 (80.0%)</td>
<td>5</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>$Ehd1^{+/-}$</td>
<td>$Ehd1^{+/-}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>5*</td>
<td>$Ehd1^{R398W}$</td>
<td>$Ehd1^{R398W}$</td>
<td>32 (19.2%)</td>
<td>88 (52.7%)</td>
<td>47 (28.1%)</td>
<td>167</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

n denotes the number of breeding pairs; some breeding pairs produced multiple litters (*).

$Ehd1^{R398W/R398W}$: Knockin mice homozygous for the $Ehd1$ mutation R398W.

$Ehd1^{R398W/wt}$: Knockin mice heterozygous for the $Ehd1$ mutation R398W.

$Ehd1^{wt/wt}$: Littermates of knockin mice without the mutation R398W.

% was calculated for each genotype based on total genotyped pups for each breeding scheme.

Homozygous $Ehd1^{+/-}$ and $Ehd1^{R398W/R398W}$ male mice were bred for at least two months with at least one heterozygous or wildtype fertile female mouse. These breeding pairs produced no offspring indicating that male homozygous $Ehd1^{+/-}$ and $Ehd1^{R398W/R398W}$ mice were infertile.
Supplemental Figures

Supplemental Figure 1: EHD1 in human kidney

Localization of EHD1 (green) in normal human kidney. Aquaporin-2 is labeled in red. The middle panel and lower panel are magnifications of the areas marked in the upper panel. Strong EHD1-positive labeling was present in proximal tubules. Scale bar 500 µm.
Supplemental Figure 2: Ehd1 in mouse kidney

Similar to the localization in human kidney, Ehd1 was found in the apical/subapical compartment of proximal tubules using antibody staining (upper left panel) or RNAscope (upper right panel). In kidney cortex of a knockout mouse (lower left panel) no Ehd1-specific antibody staining was observed while knockin mouse showed limited granular staining (lower right panel). Scale bars 100 µm.
Supplemental Figure 3: Localization Ehd1, Megalin and reabsorbed β2-microglobulin

Ehd1 and Megalin show an overlapping subcellular localization. (A) Murine Ehd1 (green) was immunostained in the kidney of a mouse that was injected with labeled β2-microglobulin (red) 30 min prior to fixation. Please note localization of β2-microglobulin and Ehd1 in the apical/subapical compartment. (B) In a consecutive section (lower panel), Megalin (green) is also localized in the apical/subapical compartment. Scale bars: 50 μm.
Supplemental Figure 4. Zebrafish

Expression analysis of zebrafish ehd orthologues and a role for Ehd1 in proximal tubular uptake. (A) Homology at the protein levels between human (Hs) EHD1 and the zebrafish (Dr) Ehd proteins. (B) RT-PCR analysis of tissue expression of zebrafish ehd transcripts. Shown is a representative PCR of tissue samples prepared from a total 18 animals, 3 biological replicates. (C) Q-PCR analysis of zebrafish ehd1a, ehd1b and ehd3 expression in the adult zebrafish kidney. The Q-PCR was performed in duplicate, each time from samples prepared from 3 biological replicates. Values are normalized to the housekeeping gene eif1a. (D) Western blot of protein extracts from 3 dpf uninjected wildtype or ehd1a-null larvae or wildtype larvae injected with splice-blocking morpholinos to ehd1a, ehd1b, or both ehd1a and ehd1b. Ehd1a and b were detected with anti-EHD1 antibody. GAPDH is a loading control. Shown is a representative Western blot of samples prepared from approximately 100 larvae per condition. (E) Uptake of Alexa488-conjugated 10 kDa dextran into the pronephric tubules of 4 dpf zebrafish larvae treated with control morpholino (MO) or morpholinos targeting ehd1a and ehd1b. Uptake was assessed at 2.5 h post-injection by fluorescence stereomicroscopy and is scored as indicated. The bars correspond to the relative abundance of the different categorized phenotypes shown in the microscopy images (black, no uptake; grey, low uptake; white, high uptake). Data are presented as the mean ± SEM. Statistical analysis was performed using the Pearson’s chi-squared test. **** indicates p ≤ 0.0001.
Supplemental Figure 5: Localization of Ehd1\textsuperscript{R398W} in mouse kidney

(A) In kidneys of homozygous Ehd1\textsuperscript{R398W/R398W} knockin mice, Ehd1-specific immunostaining was sparse and mainly localized in small aggregates within the proximal tubular cells (arrowheads). In distal segments such as thick ascending limbs, filament-like structures were found at the apical side (arrows). These Ehd1-positive apical structures were absent in wildtype mice and negative for acetylated tubulin.

(B) Protein abundance determined by mass spectrometry of kidney lysates of wildtype and Ehd1\textsuperscript{R398W/R398W} mice revealed reduced Ehd1 protein abundance in knockin mice (n=3 each group).

(C) No overlap between Ehd1-positive structures (green, arrowheads) and acetylated tubulin (red, arrows).
Supplemental Figure 6: Localization of Ehd1 and Arf6 in mouse kidneys

(A) Localization and abundance of Arf6 (red), a factor involved in trafficking of biological membranes and protein cargo, appeared to be similar in wildtype mouse kidney (upper panels), Ehd1 knockout (middle panels) and $Ehd^{1^{R398W/R398W}}$ knockout mice (lower panel). Ehd1 (green); Phalloidin (magenta) was used as a marker of the brushborder membrane; nuclei were stained with DAPI (blue). Left three panels: confocal images; right panel: STED image for Ehd1 and Arf6. Deconvolution of all images using Huygens software. Scale bar: 5 µm. (B) Relative protein abundance of Megalin, Cubilin, Amnionless (Amnls), and Arf6, determined by mass spectrometry of kidney lysates of wildtype (white bars) and $Ehd^{1^{R398W/R398W}}$ mice (gray bars). n=3 each group.
Supplemental Figure 7: Effect of Ehd1 knockout and knockin on the localization of Rab11

Localization and abundance of Rab11, a regulator of intracellular membrane trafficking routes, appeared to be similar in wildtype mouse kidney (upper panels), Ehd1 knockout (middle panels) and Ehd1^{R398W/R398W} knockin mice (lower panel). Phalloidin (green) was used as a marker of the brushborder membrane; nuclei were stained with DAPI (blue). Left two panels: confocal images; right panel: STED image for Rab11. Deconvolution of all images using Huygens software. Scale bar: 5 µm.
Supplemental Figure 8: Intravital multiphoton microscopy

(A) Original intravital microscopy experiments illustrating the delayed reabsorption of labeled β2-microglobulin (magenta) in the kidney of an Edh1−/− mouse. Blood vessels are stained by FITC-coupled high molecular dextran (green). Scale bars 75 µm. (B) Summary of experiments (6 animals each group, 31-36 tubules per group) as shown in (A) to illustrate the time course of the reduced rate of reabsorption. The data at 10 min are identical to the data shown in Figure 3G. Asterisks indicate p ≤ 0.05.
Supplemental Figure 9: EHD1 and MICAL-L1 in EDH1-overexpressing LLC-PK1 cells

A MICAL-L1  EHD1  Overlay

B MICAL-L1  EHD1<sup>R398W</sup>  Overlay

Immunofluorescence microscopy of LLC-PK1 cells expressing wildtype human EHD1 (A) or the EHD1<sup>R398W</sup> mutant (B). MICAL-L1 (red) colocalized with EHD1 (green) in cells expressing wildtype and mutant EHD1. In the overlay images, cell nuclei were stained with DAPI (blue). Cells were induced for 48 h with doxycycline (1 mg/l). Scale bar: 10 µm.
Supplemental Figure 10: MICAL-L1 and Pacsin 2 in cells overexpressing EDH1\textsuperscript{R398W}

Immunofluorescence microscopy of LLC-PK1 cells expressing the EDH1\textsuperscript{R398W} mutant, labelled with antibodies to EHD1 and either Pacsin 2 (A) or MICAL-L1 (B) to label recycling endosomes. In the overlay images, EHD1 is shown in green, Pacsin 2 and MICAL-L1 in red. Arrowheads indicate colocalization within tubules. Cells were induced for 24 h with doxycycline. Scale bar: 20 µm.
Supplemental Figure 11: Primary cilia in murine and human kidneys

Proximal tubules of wildtype (A), homozygous Ehd1 knockout (B), and R398W knockin mice (C) had similar numbers of Arl13b-positive primary cilia (arrow heads). Scale Bar 20 µm. Electron microscopy (EM) of primary cilia (arrows) within human renal proximal tubules. EM normal kidney (D) / (F); EM affected individual (E) / (G). Please note, (D) and (E) show the base of cilia (longitudinal slice); (F) and (G) show a cross section of cilia. Scale Bars 500 nm.
Supplemental Figure 12: Alignment of EHD1, EHD2 and EHD4

Multisequence alignment of human EHD1 (Q9H4M9), EHD2 (Q9NZN4) and EHD4 (Q9H223) by the program Clustal Omega reveals an overall identity between these isoforms of > 70%. The topology based on crystal structures of EHD2 (pdb entry code 4CDI) and EHD4 (pdb entry code 5MTV) is shown on top and bottom of the respective sequences. EHD proteins consist of an N-terminal unordered domain (purple), a small helical domain α1 (dark cyan), the GTPase domain comprising α2 - α5 (red), several β-sheets (indicated as black arrows) and the dimerization interface helix α6. Adjacent to the GTPase domain is the helix pair α7 - α8 (dark cyan) separated by the hinge residue Pro286 (black star) and the membrane interacting helices α9 - α11 (pink). Helix α12 connects to the EH-domain (not shown) via a long unordered linker loop. The mutation Arg398Trp in EHD1 is located at the very end of α12 (white star).
Homology models of EHD1 determined by the program Modeller using the autoinhibited state of EHD2 (pdb entry code 4CDI) (A and B) and the activated state of EHD4 (pdb entry code 5MTV) (C and D) as a template, respectively. A and C: Similar to EHD2 and EHD4, EHD1 presumably forms stable dimers (monomers are colored in red and grey) with α6 serving as dimerization helix (box in dotted line). EH-domains are only resolved in the auto-inhibited state locking the ATPase domain of the neighboring monomer in the dimer. Although the EH-domain of EHD4 was present during crystallization it was not resolved suggesting that the blocking interaction was released during activation. Since EH domains were not resolved in the activated state, they are not shown in (C) and (D). The higher degree of flexibility is in agreement with the functional role of EH-domain as interaction partners for different proteins in the activated state. The domain structure in the EHD1 homology model is shown in the putative autoinhibited state (B) and the activated state (D). Arg398 is shown in sphere representation located at the end of α12. The KPF loop is only ordered in the activated state (colored in gold in (D)).
Supplemental Figure 14: Structural modeling: Effects of the R398W mutation on EHD1 oligomerization

Mutation of Arg398 against a bulky residue such as tryptophan probably disturbs the dimer-dimer interaction between the KPF loop and the helical bundle and thereby prevents oligomerization. A possible pathological interaction partner of Trp398 suggested from homology modelling would be Arg287, which would strengthen the interactions between $\alpha_{12}$ and $\alpha_8$ within one monomer.
Supplemental References


A Founder Mutation in EHD1 Presents with Tubular Proteinuria and Deafness

**METHODS**

Homozygous R398W mutation of the membrane-shaping protein EHD1 in six patients with tubular proteinuria and deafness.

Disease mechanisms were analyzed using genetics, cell biology, structural biology, zebrafish and mouse models.

**OUTCOME**

Conclusion

Based on six patients with proteinuria and hearing deficit, EHD1 was identified as a critical component of the renal protein reabsorption machinery and inner ear function.

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