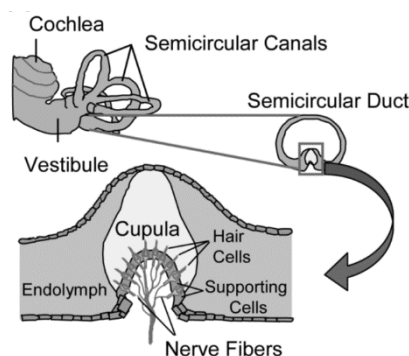*Supplementary Materials:*



**Supplementary Figure S1.** Schematic representation of the localization of the cupula in the (human) inner ear [1]. Figure reused with permission from Ref. [1].



**Supplementary Figure S2.** Visual multiple sequence alignment using CLUSTAL W (https://www.genome.jp/tools-bin/clustalw) [27]. Zebrafish (accession A0A0R4ITH5 ), salmon (accession ACN10635, ~86.4% identity) and human (accession AAH31261, ~71.5% identity) ZPLD1 amino acid sequences are highly conserved.
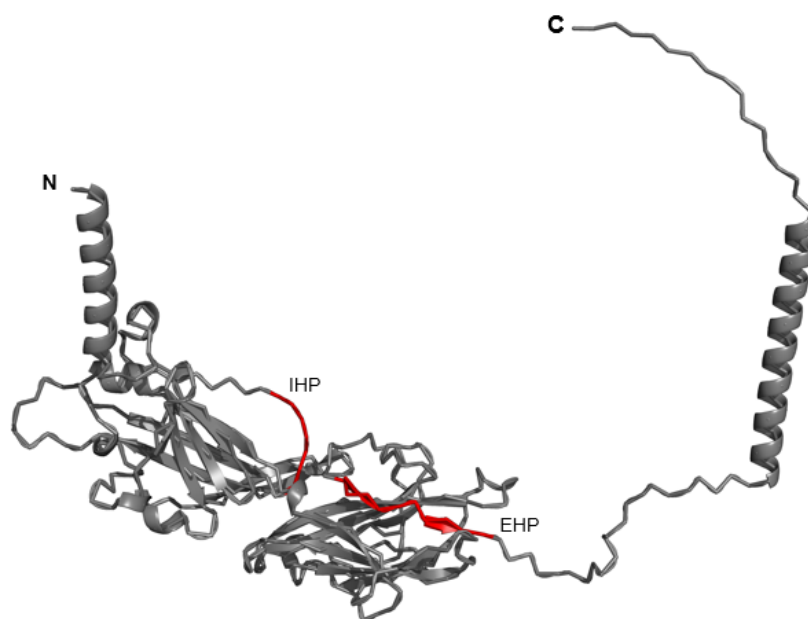
**Supplementary Figure S3.** Visualization of predicted structure of zebrafish ZPLD1 (accession A0A0R4ITH5). Model prediction by AlphaFold (AlphaFold DB version 2022-06-01) [33,34]. Putative IHP and EHP are marked in red.
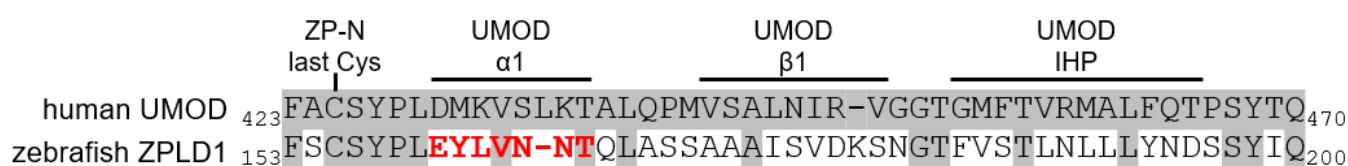


**Supplementary Figure S4.** Sequence comparison of the interdomain linker sequences of zebrafish ZPLD1 (accession A0A0R4ITH5) and human Uromodulin (accession P07911). The structured rigid linker region of Uromodulin consists of an $\alpha$-helix ($\alpha$1), a beta-strand ($\beta$1) and the consecutive IHP [21]. Alignment reveals that Schaeffer´s proposed model for the prediction of the IHP as well as the relevant hydrophobic sequence we identified for ZPLD1 (marked in red) might correspond to the $\alpha$1-helix rather than the internal hydrophobic patch.

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**TTTGCCCGGTGCTTTTTTCTGGCTACACTGACACTGACCTGGCACTGAATGGGCGTCACGGGGATGCCCACTGCAGAGGCTTCATCAACAACAACACGTTCCCCACGGT**
**TGTGCTGTTTAGCATCAGTCTCAACACTCTGGAGTCTTGCGGAAACTCACTGGTGGTCTCTACGGCTCATGGGCCCAACGCCTATGGGAATCTTTCCCTGGTCCAGATT**
**GGGAATATAGCAGGATACATTGACACTCCGGATCCCCGACTGTCATCAGTTACCTACCAGGACTGCTTTACAAGTTCAGTTGTAGCTACCCACTGGAATACCTGGTCA**
**ACAACACACAGCTTGCTTCGTCGGCCGCAGCGATATCAGTGAAGGACAGTAATGGTACTTTTGTTAGCACGCTCAATTTGCTTCTATACAACGACTCCTCATACATTCA**
**GAACCTCGCCATCCCAATGTCTGGACTTACTCTGAAGACGCGAGTGTTCGCCGCTGTAAAAGCCACCAACCTAGATAGGAGGTGGAACGTTCTGATGGACTACTGTTAC**
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**GCTTCTCCTTTGAGGTGTTTCGCTTTGTCAAGCACAAAAACCAGAAAATGTCGACTGTTTTTCTGCACTGCGTGACCAAGCTGTGTCGCTCTGATGACTGCCCCATGCT**

**CCTGCCCATCTGCGGTAAGAGGAAGAAAAGGGATGTGTCAGAAAGAAGCGGTGGTGCCTCAGACAATGCAGTTATAACCGCTGGACCCATCATCACCCGAAGTGATGAA**

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**Supplementary Figure S5.** Full sequence of initially ordered expression vector (accession A0A0R4ITH5). The zebrafish ZPLD1 sequence is indicated in bold letters.

**Supplementary Table S1.** Primers (5′-3′) used to generate ZPLD1 expression constructs

| Mutation | Primer |
|---|---|
| Deletion of eGFP | Forward: CAGCCATACCACATTTGTAGAGGTTTTACTTGC |
| | Reverse: TCACTCGAGGCTGAACGCTGGG |
| Insertion of the Strepavidin-tag (and corresponding Linker) | Forward: TGGAGCCATCCGCAGTTTGAAAAAAGGAA-GAAAAGGGATGTGTCAGAAAGAAGCGG |
| | Reverse: AGAGCCAGAAGACTTACCGCAGATGGGCAGGAGCATGGGGC |
| Deletion of the putative EHP | Forward: AGTGATGAAACTCCCAGTAATATATCCC |
| | Reverse: TGCATTGTCTGAGGCACCACCGC |
| Deletion of the putative IHP | Forward: CAGCTTGCTTCGTCGGCCGCAGCG |
| | Reverse: CAGTGGGTAGCTACAACTGAACTTGTAAAGCAGTCC |
| Deletion of the putative TMD | Forward: ATGTCGCTGCTGAGGGGAAAACAGACG |
| | Reverse: TGAATTCATTTTGAATGGAGGGCCG |
| Insertion of the N-terminal 8xHis-tag (N-His) | Forward: TCTTCTGGCTCTTTCAATGGGTTCAACTGTGATGCCAACTTCC |
| | Reverse: ATGATGATGATGGTGATGGTGATGTTGAGCATTCGC-TATGAAAGTTTTACTTACAAGC |
| Insertion of the C-terminal 8xHis-tag N-terminal of the EHP (C-His1) | Forward: CATCATCATCATGGTGGTGCCTCAGAC |
| | Reverse: GTGATGGTGATGGCTTCTTTCTGACACATCCC |
| Insertion of the C-terminal 8xHis-tag C-terminal of the EHP (C-His2) | Forward: CATCATCATCATAATATATCCCAACTAGCGCAG |
| | Reverse: GTGATGGTGATGACTGGGAGTTTCATCACTTCG |
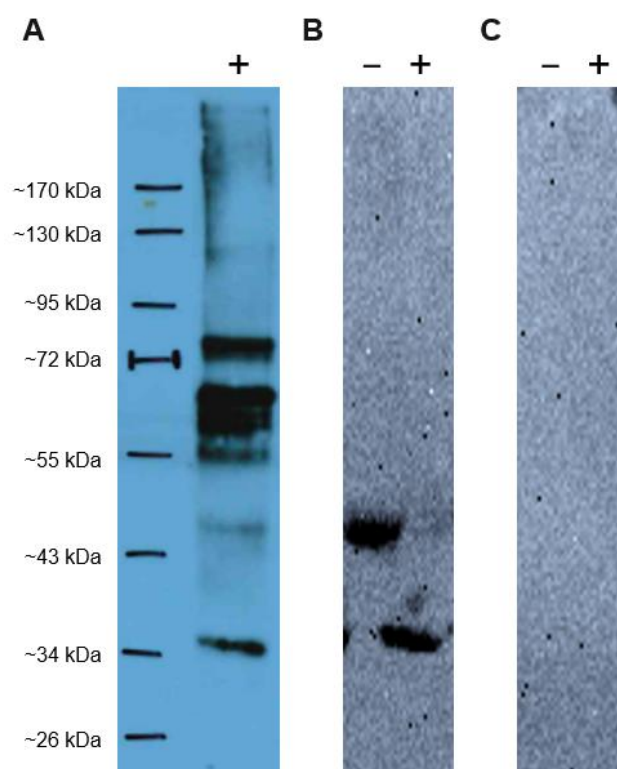
**Supplementary Figure S6.** Western blot detecting of PNGase F treated (+) as well as untreated (-) ZPLD1. The protein was purified from MDCK cell culture media. (A) Protein reduced and denatured in Laemmli buffer containing 2.5% mercaptoethanol at 96 °C for 5 min Besides the 35 kDa monomer higher molecular weight species can be observed. (B) Protein reduced and denatured in Laemmli buffer containing 5% mercaptoethanol at 96 °C for 15 min to obtain a higher portion of monomeric protein. (C) Control sample obtained from mock transfected MDCK cells.
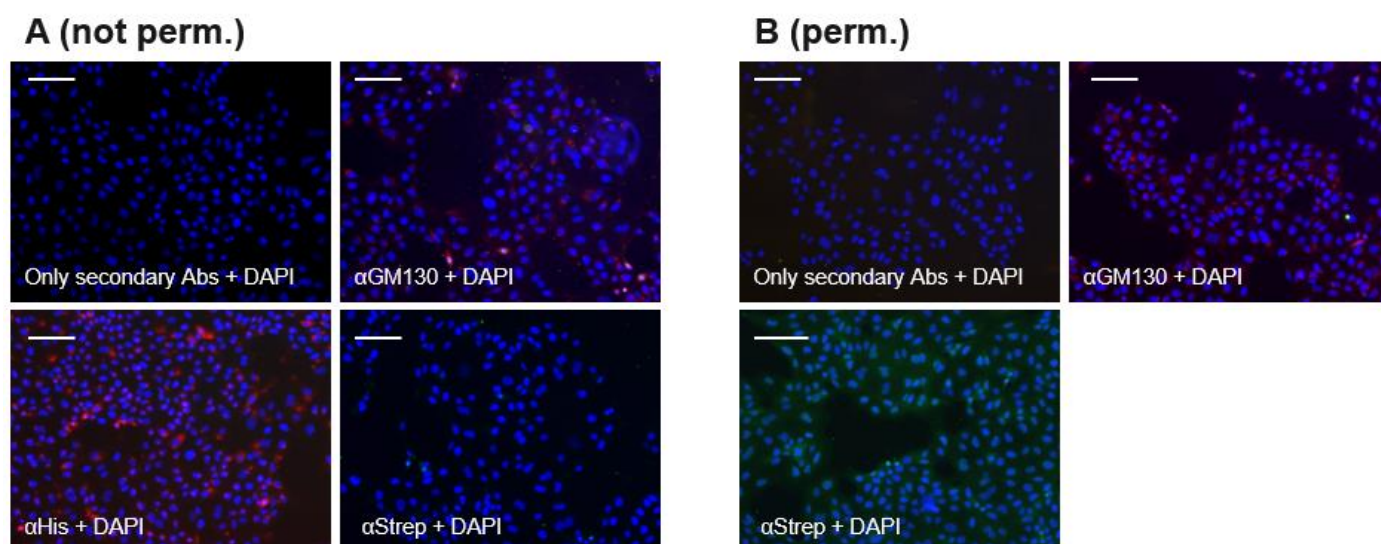


**Supplementary Figure S7.** Control immunofluorescence images of untransfected MDCK cells. Cells treated with: a mixture of all used secondary antibodies, antibodies against the Golgi marker GM130, and tag antibodies (His/Strep) (see Materials and Methods). Alle samples were additionally stained with DAPI. (A) Cells were not permeabilized; (B) Cells permeabilized using Triton X-100. Bar, 50 μm.