Supplementary material 1. (A) This clinical note presents a female patient with a homozygous *TBCE* variant and a compound heterozygous *GJB2* variant *(i)*. To investigate their potential synergy, we used in *silico* protein modeling and molecular dynamics simulations (MDS) to reproduce mutant proteins and predict structural and functional changes *(ii-iii)*. This approach helps correlate genotype with phenotype, offering insights into the complex genetic landscape of syndromic presentations.

(A) *(i) Clinical exome sequencing*

DNA extraction from peripheral blood samples of parents and the child was collected to perform clinical exome sequencing (CES). Genomic DNA libraries and CES were performed using the CE-IVD SOPHiA DDM Clinical Exome Solution v3 (pipeline ILL1XG1G5_CNV_exome_NextSeq) (SOPHiA Genetics, Switzerland) for sequencing on NextSeq 550DX Illumina platform (Illumina, USA) following the manufacturer's recommendations. Data were analyzed with the SOPHiA DDM software (v.10.40), using hg19 (GRCh37) as reference, for the generation of binary alignment map and variant call format files, single nucleotide polymorphism annotation, as well as indel and CNV annotation within the coding regions and exon-intron boundaries (+/- 5 bp) of the genes of interest. The panel of investigated genes (n=50), listed alphabetically, includes: *AIP, AIRE, ATP6V0A4, ATP6V1B1, BSND, CA2, CACNA1D, CACNA1H, CACNA1S, CASR, CHD7, CLCN1, CLCNKA, CLCNKB, CLDN16, CLDN19, CNNM2, CYP24A1, EGF, FXYD2, GATA3, GCM2, GNAS, GJB2, HADHA, HADHB, HNF1B, HNF4A, HSD11B2, KCNA1, KCNJ1, KCNJ2, KCNJ5, MAGT1, PCBD1, PDEAD, PRKAR1A, PTH, SARS2, SCN4A, SCNN1B, SLC12A1, SLC12A3, SLC26A3, SLC34A1, STX16, TBCE, TBX1, TBX2, TRPM6*.

The test's specificity and sensitivity exceed 99%; quality indicators, such as average coverage of 30X and Phred Quality Score=30 in regions of interested. Genetic variants were annotated according to the Human Genome Variation Society nomenclature and classified according to the American College of Medical Genetics and Genomics guidelines.[1] Data interpretation was carried out employing public databases such as ClinVar (www.ncbi.nlm.nih.gov/clinvar/), VarSome (varsome.com/), Franklin (franklin.genoox.com), MutationTaster2021,[2] as well as relevant scientific literature.

(A) *(ii) Protein-protein interaction analysis*

The tool STRING (Search Tool for Retrieval of Interacting Genes/Proteins) (string-db.org/) version 12.0 was employed to seek potential interactions between *TBCE* and *GJB2*. The protein-protein interaction network was constructed applying as active interaction sources text mining, experiments, databases, neighborhood, gene fusion, co-occurrence and co-expression, as well as species limited to "*Homo sapien*s" and an interaction score>0.4.

(A) *(iii) Molecular dynamics simulations*

In order to perform MD simulations, the crystal structures of the 2 proteins TBCE and Cx26 were retrieved from the protein data bank (PDB).[3] For TBCE, the computed structure model was employed (code AF_AFQ15813F), and for Cx26 the crystal structure was used (PDB code 2ZW3, resolution 3.29 Å).[4,5] Flare software (version 8.0; Cresset®, Litlington, Cambridgeshire, UK) was used to perform in silico mutagenesis of the proteins.[6,7] The "mutate to" function implemented in Flare software was used to mutate the amino acids that are associated to the pathology to create the different protein variants. To increase the stability of the system and decrease steric clashes, the obtained proteins underwent 3 dynamic relaxation cycles with a duration of 10 ns. These relaxation cycles were performed under number of particles, volume and number of particles, pressure, temperature conditions (T=300 K; P=1.01325 bar) using NAMD 2.14 software.[8] Consequently, the most stable system was chosen for MDS.

Cx26 was embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine phospholipid bilayer using CHARMM-GUI, and its arrangement within the membrane was aligned using the orientations of proteins in membranes database.[9-11] AmberTools were utilized to solvate the membrane-protein system, which was subsequently neutralized by the addition of Na+ and Cl- atoms. The force field for the proteins was set to AMBER FF14SB, while TIP3P was employed for the water model.[12,13] To conduct the MDS, NAMD 2.14 software was employed.[8] To reduce steric clashes of the protein within the membrane, minimization was carried out using short dynamic cycles, specifically 5 ns, under NVT and NPT conditions. Five dynamic simulations were conducted for a duration of 50 ns following the protocol in Falsaperla et al.[14] The analysis conducted at the 50 ns, in conjunction with the root-mean-square deviation (RMSD)

evaluation, did not reveal any substantial alterations. The dynamics files were visualized and assessed using VMD software (ver. 1.9.4).[15] To assess significant differences in RMSD between wild-type and mutant proteins, we ran 5 independent 50-ns simulations per variant and focused on the final 20 ns (where convergence was reached). We divided this window into four 5-ns blocks, calculating the mean and standard deviation of the RMSD in each block to reduce autocorrelation effects. This produced 20 RMSD data points per system, which were tested for normality (Shapiro-Wilk) before applying a 1-way analysis of variance and Tukey *post hoc* test to identify significant differences in structural stability.

(B) Summary of genetic variants identified by clinical exome sequencing in the proband.

Gene	Variant	Chr.	Exon	Pattern of inheritance	ACMG class	gnomAD (%)	ClinVar ID
TBCE (NM_003193.5)	(c.155-166del; p.Ser52_Gly55del)	1q42.3	3	HMZ	5	0.006	5290
GJB2 (NM_004004.6)	rs803389939 (c.35del; p.Gly12Valfs)	13q12	2	HTZ (maternal)	5	0.0062	17004
	rs72474224 (c.109G>A; p.Val37Ile)			HTZ (paternal)	5	0.0076	17023

ACMG, American College of Medical Genetics and Genomics; Chr., chromosome; HMZ, homozygosity; HTZ, heterozygosity.

Supplementary references

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