SUPPLEMENTAL DATA

PSENEN mutations underlie Dowling-Degos disease associated with acne inversa

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Supplemental methods

Patients and families

The analyses included patients/families with the typical clinical and histopathological features of DDD. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid and DNA was extracted from peripheral blood leukocytes according to standard methods. Ethical approval was obtained from the ethics committees of the Medical Faculties of the University of Düsseldorf and the University of Bonn, respectively. All participants provided written informed consent prior to blood sampling. The study was conducted in concordance with the Declaration of Helsinki principles.

Exclusion of mutations in *KRT5*, *POFUT1* and *POGLUT1*

Initially, mutations in the three known DDD-genes namely *KRT5*, *POFUT1* and *POGLUT1*, had been excluded by Sanger-sequencing. Amplicons covering all coding exons and exon-intron boundaries were generated using standard polymerase chain reaction with primers depicted in Supplemental Tables 1-3. Sanger sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3100 genetic analyzer (Applied Biosystems). Data were analyzed using SeqMan II software (DNASTAR).

Whole-exome sequencing and confirmation of mutations

Whole-exome sequencing to identify the underlying genetic defect in the patients studied here was performed in eight affected individuals. Six individuals originate

from a large Indian family with ten affected individuals across four generations (Supplemental Figure 1A). Another individual selected for exome sequencing originates from a large Thai family with 12 affected individuals over three generations (Supplemental Figure 1B). The last individual selected for exome sequencing was a simplex case from Germany without other affected relatives.

For whole-exome sequencing, 1 µg of DNA was fragmented with sonication technology (Bioruptor, Diagenode). The fragments were end-repaired and adaptor-ligated, including incorporation of sample index barcodes. After size selection, we subjected a pool of all 5 libraries to an enrichment process with the SeqCap EZ Human Exome Library version 2.0 kit (Roche NimbleGen). The final libraries were sequenced on an Illumina HiSeq 2000 sequencing apparatus (Illumina) with a paired-end 2×100 bp protocol. This resulted in 5.7 - 9.1 Gb of non-duplicated mapped sequences (on average 7.2 Gb), a mean coverage of 78 - 125X (on average 97X) and 30X coverage of 87 - 94% (on average 90%) of the target sequences. The Varbank pipeline v.2.1 and 2.7 were used for data analysis and filtering as previously described elsewhere (1). Data were filtered for high-quality rare (MAF < 0.005) autosomal variants affecting protein structure or splice sites and attention was focused on genes with the highest burden of functional variants in our individuals.

Sequence variants detected and considered to reflect disease causing mutations were confirmed by Sanger sequencing using the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI 3100 genetic analyzer (Applied Biosystems). Screening of our cohort for additional mutation in *PSENEN* was performed by usage of Sanger sequencing (Supplemental Table 4).

Cell line

The HEK293T cell line was kindly provided by Thomas Zillinger (Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn). HEK293T is a distinct derivative of HEK293 cell line listed in the ICLAC database. It contains a transgene expressing the SV40 large T antigen and is morphologically distinguishable from the HEK293 cell line. The HEK293T cell line used in this study is authenticated by the competence to replicate vectors carrying the SV40 origin of replication by regular use for high titer lentivirus production at the Institute of Clinical Chemistry and Clinical Pharmacology. HEK293T cell lines were tested for mycoplasma contamination and confirmed to be mycoplasma free. Cells were maintained at 37°C (5% CO_2) in DMEM (Lonza) supplemented with 10% FCS (Life Technologies), 1% Penicillin-streptomycin (10,000 U ml-1, Life Technologies) and 1% Amphotericin B (250 µg ml-1, Life Technologies). Cells were cultured in 10 cm Petri dishes.

Exon trapping

To analyze the structural consequences of splice site mutation c.62-1G>C we chose an exon trapping strategy. Accordingly, we cloned genomic DNA containing this mutation into the Exon Trap vector (MoBiTec, Supplemental Table 5) and transfected HEK293T cells from which we isolated mRNA 48 h post-transfection using Dynabeads mRNA DIRECT Micro Kit (Life Technologies). After reverse transcription of mRNA into cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems), Sanger sequencing was performed as described above.

Analysis of nonsense and frameshift mutations by epitope tagging

To study the consequences of mutations p.Arg39* and p.Ser73Profs*72, *PSENEN* coding sequences was cloned into the TOPO cloning site of pcDNA 3.1/V5-His-TOPO vector (Invitrogen, Supplemental Table 5) according to the manufacturer's protocol. The mutant constructs (p.Arg39* and p.Ser73Profs*72) were generated by targeted mutagenesis using the QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) according to manufacturer's instructions (Supplemental Table 6).

All constructs were verified by Sanger sequencing. HEK293T cells were transiently transfected with the plasmids by use of Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Subsequently, cells were collected 48 h post transfection and immunoblot analysis was performed using a rabbit anti-FLAG primary antibody (Sigma Aldrich #F7425, 1:1000) and a mouse anti-GAPDH primary antibody (Sigma Aldrich G8795#; 1.2000).

Zebrafish studies

Zebrafish experiments comply with actual animal protection laws and animal research husbandry regulations. A §11 husbandry license by the veterinarian office in charge (City of Bonn, Germany) is in place under file reference 56-2. All research experiments involving zebrafish were carried out in accordance with the approved national guidelines and were performed on zebrafish larvae ≤5 dpf (days post fertilization).

For this study, we used WT (TL/EKW and TU/TL mixed background) zebrafish larvae ≤ 5 dpf, all from parents negative for the leot1 tupfel mutation. Original TL/EKW WT strain fish were a gift from the M. Hammerschmidt laboratory (University of Cologne,

Germany); the TU/TL WT strain fish were obtained from the European Zebrafish Resource Centee (EZRC, Karlsruhe, Germany). All zebrafish were housed in our fish facility conforming to national regulations. Fish were kept at 28ºC in system-water at 550 µS and a pH of 7.2 under a 14-hour light/10-hour dark cycle. Zebraish were fed 3 times a day with dry pellet food (GM) and live brine-shrimp. Fish eggs were obtained by natural spawning in the morning at light onset.

Knockdown of *PSENEN* homolog *psenen* in zebrafish

To provide in vivo support for PSENEN as a DDD disease gene, we performed knockdown of *PSENEN* homolog *psenen* in zebrafish larvae using antisense morpholinos® (Gene Tools, Supplemental Table 7). Fertilized fish eggs were obtained from natural spawning of wild type adults (TL/EKW) in our aquaculture facility, which is maintained according to current animal regulations. 2 ng of pen-2 antisense morpholinos directed against the ATG (MO1) or 5'- untranslated region (MO2) respectively were injected into fertilized zebrafish eggs in between the one- to four-cell stage. Hereto we followed the protocol and design of the morpholinos previously described by Campbell et al. in 2006 but omitted 1-phenyl 2-thiourea (PTU) in order to study pigmentation of the larvae (2). Because the aforementioned study showed that a lack of pen-2 induces p53-dependent apoptosis in the zebrafish larvae, 4 ng of a p53-rescue-morpholino (p53MO) was also co-injected as described by Campbell et al. in parallel to prevent this happening. As a control, embryos were injected with 2 ng of a morpholino harboring the corresponding sequence in a scrambled order (CoMO1/CoMO2) plus p53MO. As an additional control, a non-treated (injected) littermate group was used. Adequate knockdown of pen-2 was confirmed by immunoblotting using a rabbit anti-pen-2 primary antibody (Acris #

AP22868PU-N, 1:1000) and a mouse beta-actin antibody (Sigma # A3854, 1:10000).

At 72 and 96 hours post fertilization (hpf) pictures of the zebrafish larvae were taken under a Nikon AZ100 continuous 8x zoom-microscope to study melanin content and distribution.

In vivo imaging of pigment cells' migration and differentiation

To monitor the migration and differentiation of pigment cells in the developing zebrafish larvae, we carried out in vivo imaging. Hereto, at 80 hpf, zebrafish embryos were mounted in 1.5 % low melting point agarose, two zebrafish larvae per well in a 12-well glass bottom plate, covered with embryo medium containing 0.16 g/l concentration MS-222 and a Leica inverse-microscope DMIRE2 with a Leica DFC350 FX camera was programmed to take time lapse pictures of the developing tail region every 20 min using the Leica FW4000 (v1.2.1) software system. Data were obtained and collected during a period of 24 h hours (80 hpf to 104 hpf). Pictures of every single zebrafish larvae were assembled to a time-lapse stack respectively. After image correction for horizontal and vertical drift as well as growth of the whole zebrafish larvae (StackReg registration, Image J analysis software), migration of single pigment cells was tracked using the tracking tool from Image J analysis software. A total 44 cells from seven *psenen* knockdown larvae (MO1) and 54 cells from 9 control-injected larvae (CoMO1) from two independent experiments were analyzed. The migration behavior of single individual cell movement steps was visualized in a rose plot using the Chemotaxis and Migration tool 2.0 from ibidi. The morphological changes and differentiation of single pigment cells were quantified by measuring their horizontal and vertical diameter by manually fitting their shape with

an ellipse every 4 hours respectively using the NIS-Elements imaging software from Nikon.

Statistics

Data in Fig. 3C are depicted as dot plot showing the mean values together with 25 and 75 percentiles. Number of replicates in each condition or group are provided in the figure legends. After confirming Gaussian distribution by using a D'Agostinos-Perrson omnibus test, statistical analyses for the data shown in Fig. 3C were performed using one-way analysis of variance ANOVA and, thereafter, a Bonferroni post hoc test for multiple comparison. A P-value of less than 0.05 was considered statistically significant (*** $P \leq 0.001$).

Supplemental tables

Supplemental Table 1 Primers used for sequenecing of *KRT5* exons

Exon	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)	Amplicon (bp)
1a	TGCTGAGCTCTGTTCTCTCC	GATTTGGTTTCGGCGGTGGA	406
1b	GGGGCTCCAAGAGGATATCC	GTACAGTTCTGTGTTTCCGT	74
1c	ATCCTTTTTGGGCTGCTCACAGC	CCTCAACAATAAGTTTGCCTCCTTCA	379
2	GGAGGCACCTTAGTGAGTTG	GTCTACTACCATGCCTTCCT	378
3	TGGCCAGAGGTTCATGCTAC	GGAGCTGGAGGCCAAGGTTGA	371
4	GAGAACCAGCAGCCTGCAG	GCATGTCTCTGACACCTCA	336
5	GTTGTCATATCACAATGAGTG	GGCCATGACGACACTAAGAA	318
6	CACCACTTAGTACTCACTGC	GCTAAACACACGCAGCTAGA	374
7	TGACCCAGAAACTCAGAAGG	CATGTATGTGTGTTGTACACC	245
8	GAATGAACTCGAATCATGAGG	ATAACCATATGCTGGGATGG	427
9	TAAATGGGCCATGCAGGATC	CCATGGAGATTGCCTCTTCT	536

Supplemental Table 2 Primers used for sequenecing of *POFUT1* exons

Exon	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)	Amplicon (bp)
1	TCGGGCCATTGTGCGGTGC	GGTCCAGGAGAGAACCTCAGA	458
2	TTCTTATGCTGCCAGCTCCCC	CACTTAACCCTCAAGTCCTCTG	340
3	AATGTCTAAAGTAGCCACGGGG	GCAAAATGTGGGGAAAAGCACC	335
4	TTGCAGAGTGACATTTCTCCT	CCTGTGTGATCTGTTCCCAT	427
5	TGTTTCTTGCGTGTTGCAGGG	CCAGAGTGCCTGCCAGCCA	513
6	TTAGGTTCTTCCTGTATAGCC	CCTGGAGCTCACGTATCC	560
7	TGTCTGTAACTGCAGCTCAGG	CCTGGCAGCCAGAGGTGCT	375

Exon	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)	Amplicon (bp)
1	CAGCCTTCTCAGGGAAACTC	AATGGGTTACTCTCCAGGGTC	470
2	AATTCCTCCCTAGCGTTTCG	CTTGCCTTTCTGACCACTGC	339
3	GCTGCTCATTCTCACTCTCG	GGAGGGATCTGCTTGTTAGTC	384
4	GACTGATGATATGGAACACTGG	CCTCATCCTTACCATTATCCATC	365
5	GCTGACCTTTAATGTGATTCTG	AAATACTGACTGAGCACAGGG	1227
6	AATCTTCAGAAATGGTTAAAGCAC	CATCACATAATCAATGACCGAC	417
7	TTGTGCAGAGTCATTGCATTG	ACATTCACCTCACCACTGAC	319
8	TCTATTTAGAGCCACACTGAC	AGTGCATTTAGGCCCTGAGG	425
9	AACAACCTGTAGTCCTAGCC	TAGCCTACTTGGATAATAATGC	548
10	GGGTATCTTTAATCAGTGTGTGG	CCCAGGACAGGTTTAGTTCTG	400
11	TGTGGATATGATTGCCATAGG	TCAAGGTATAGGTGCCAAGC	409

Supplemental Table 3 Primers used for sequenecing of *POGLUT1* exons

Supplemental Table 4 Primers used for sequenecing of *PSENEN* exons

Exon	Forward Primer (5‘-3‘)	Reverse Primer (5‘-3‘)	Amplicon (bp)
1	GCCCACCCTAGTAAATATAAAGAG	GCCTGGATTCTTAAGTCTAAGAGAT	371
2	CTGGATCCCAAAGAGGAGC	GCTGGTCTAGGGAAGTCTGGA	412
3	GAATCTTGGTGGGAAGGGAC	CATCTGTTGAGAGCTTTGTACGAG	509

Supplemental Table 5 Primers used for cloning of *PSENEN*

Plasmid name	Primer	Primer sequence (5‘-3‘)	Restriction enzyme
pcDNA3.1 /V5-His-TOPO	FLAG-PSENEN-WT	Fw:ACCATGGATTACAAGGATGACGACGATAAGCCAGGACCAATGAACCTG GAGCGAGTGTC Rev:CCCTGACAACTTCTGCACATACT	/
pcDNA3.1 /V5-His-TOPO	FLAG-PSENEN-*39	Fw:ACCATGGATTACAAGGATGACGACGATAAGCCAGGACCAATGAACCTG GAGCGAGTGTC Rev:CCCTGACAACTTCTGCACATACT	/
pcDNA3.1 /V5-His-TOPO	FLAG-PSENEN-*72	Fw:ACCATGGATTACAAGGATGACGACGATAAGCCAGGACCAATGAACCTG GAGCGAGTGTC Rev:GTCCCACGTTCTCTGCTGA	/
pET01	PSENEN-ET	Fw:ATAGTACTCGAGAATCCAGCCAGCTTACTAGTG Rev:GATGTCAGCAGAGAACGTGGCGGCCGCTACAA	XhoI NotI

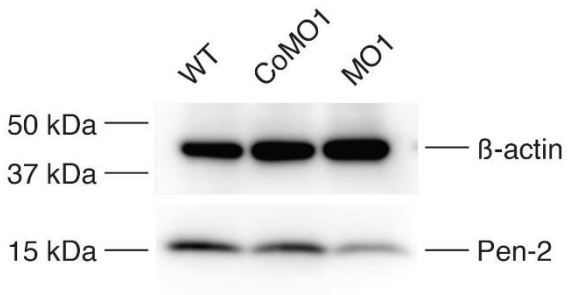
Supplemental Table 6 Primers used for mutagenesis

Primer	Primer sequence (5‘-3‘)
Mut *72	Fw: AGATGGTGATCCAGGAGTGAGCACTATCACCC
	Rev: GGGTGATAGTGCTCACTCCTGGATCACCATCT
Mut *39	Fw: GACAAGGAAGGCCTCTCAGAAGAACCAGAAGATGT
	Rev: ACATCTTCTGGTTCTTCTGAGAGGCCTTCCTTGTC

Supplemental Table 7 Morpholinos used for knockdown of *psenen* in zebrafish

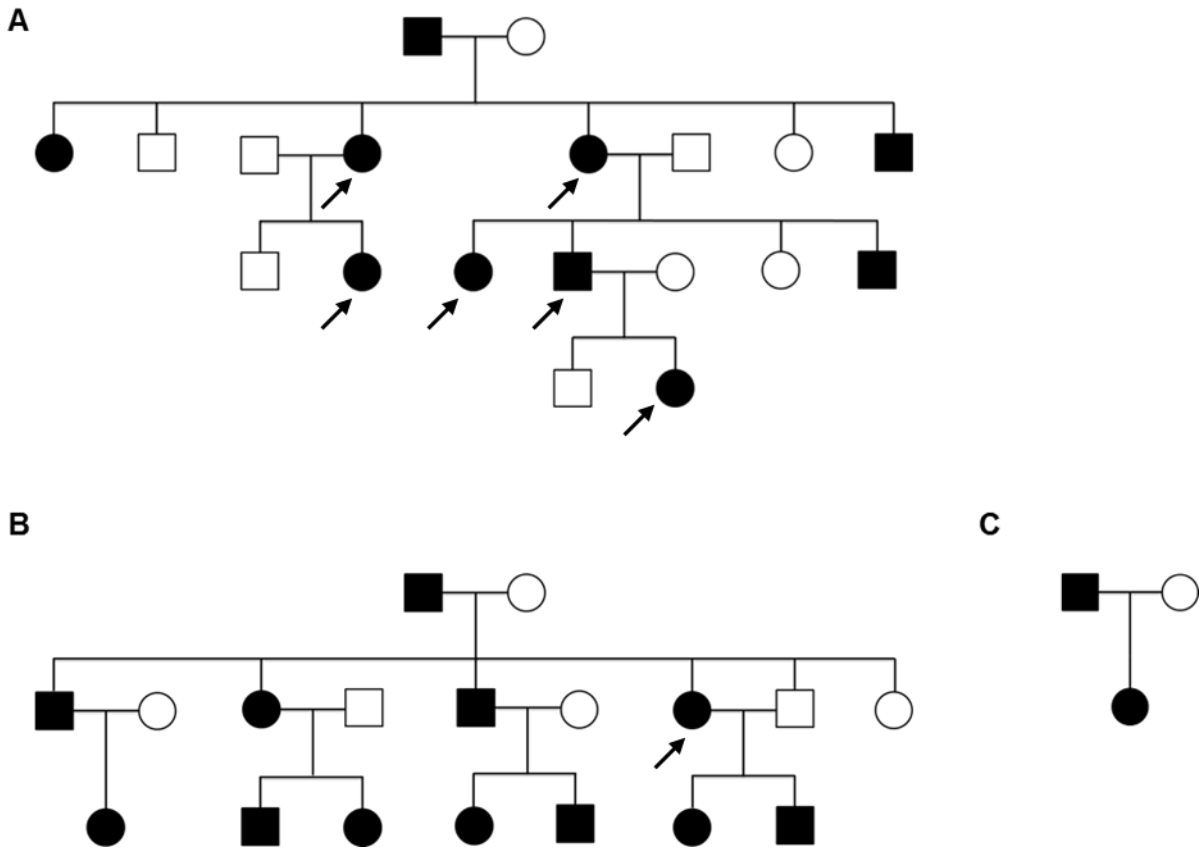
Morpholino	Sequence (5‘-3‘)
Pen2-MO1	CATAATGAAGTATTTGTGGTGGTGG
Pen2-CoMO1	GTAGTGTGGTAGACTAGTTATGGAT
Pen2-MO2	TTTCCTTAAAAGACGAGCACAGACG
Pen2-CoMO2	GCAATCTCGGCCTAAGATAGCATAA
P53-rescue-MO	GCGCCATTGCTTTGCAAGAATTG

Supplemental figures



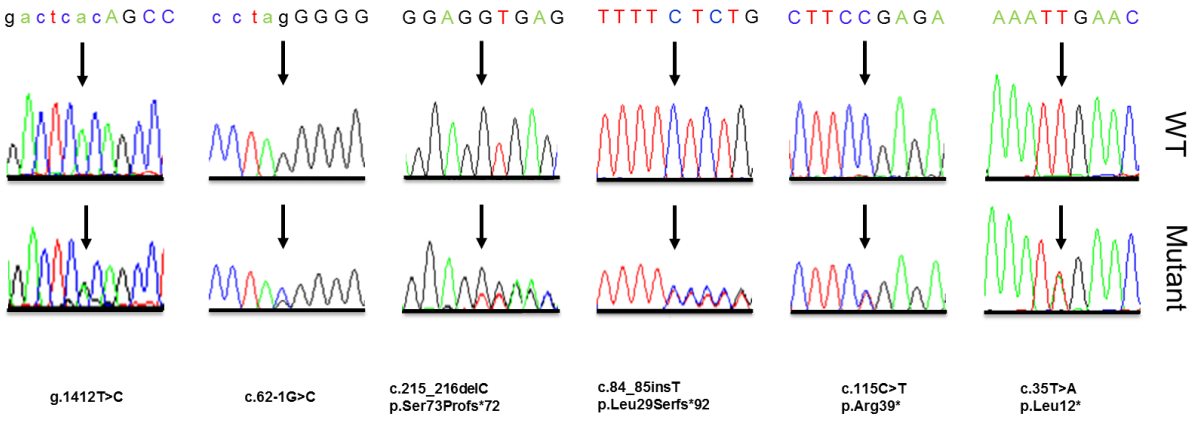
Supplemental Figure 1. Knockdown of *PSENEN* homolog *psenen* in zebrafish using MO1/CoMO1

Adequate knockdown of pen-2 was confirmed by immunoblotting.



Supplemental Figure 2. Pedigrees of DDD-families with *PSENEN*

Affected family members are shown in black; circles and squares denote females and males, respectively. (A) Pedigree of the Indian family with ten affected individuals across four generations. (B) Family from Thailand with 12 affected individuals across three generations. (C) Pedigree of the German family with father and daughter affected with DDD. Patients selected for exome-sequencing are indicated by arrows.



Supplemental Figure 3. Results of sequencing analyses in *PSENEN*: Electropherograms of all identified mutations in DDD patients.

The mutations are depicted in comparison to the corresponding wild type (WT)-sequences. Mutations are defined on both the nucleotide and protein level.

A. Wild type acceptor site

ATGAACCTGGAGCGAGTGTCCAATGAGGAGAAATTGAACCTGTGCCGGAAGTACTACCTGGgtaaggcagatc…t
ggggtttgtttgtttgtttttctttctccctagGGGGGTTTGCTTTCCTGCCTTTTCTCTGGTTGGTCAACATCTTCTGGTTCTT
CCGAGAGGCCTTCCTTGTCCCAGCCTACACAGAACAGAGCCAAATCAAAGGCTgtgagtctagagcacagaggaggga
…aggctggtctagggaagtctggagagcagccggaggccaacccttccagcttctgtttcccatgacagATGTCTGGCGCTCAGCTGTG
GGCTTCCTCTTCTGGGTGATAGTGCTCACCTCCTGGATCACCATCTTCCAGATCTACCGGCCCCGCTGGGGTGC
CCTTGGGGACTACCTCTCCTTCACCATACCCCTGGGCACCCCCTGA

B. Exon skipping

ATGAACCTGGAGCGAGTGTCCAATGAGGAGAAATTGAACCTGTGCCGGAAGTACTACCTG**G**gtaaggcagatc…t
ggggtttgtttgtttgtttttctttctccctacGGGGGTTTGCTTTCCTGCCTTTTCTCTGGTTGGTCAACATCTTCTGGTTCTTC
CGAGAGGCCTTCCTTGTCCCAGCCTACACAGAACAGAGCCAAATCAAAGGCTgtgagtctagagcacagaggagggag
…ggctggtctagggaagtctggagagcagccggaggccaacccttccagcttctgtttcccatgacag**AT**GTCTGGCGCTCAGCTGTG
GGCTTCCTCTTCTGGGTGATAGTGCTCACCTCCTGGATCACCATCTTCCAGATCTACCGGCCCCGCTGGGGTGC
CCTTGGGGACTACCTCTCCTTCACCATACCCCTGGGCACCCCCTGA

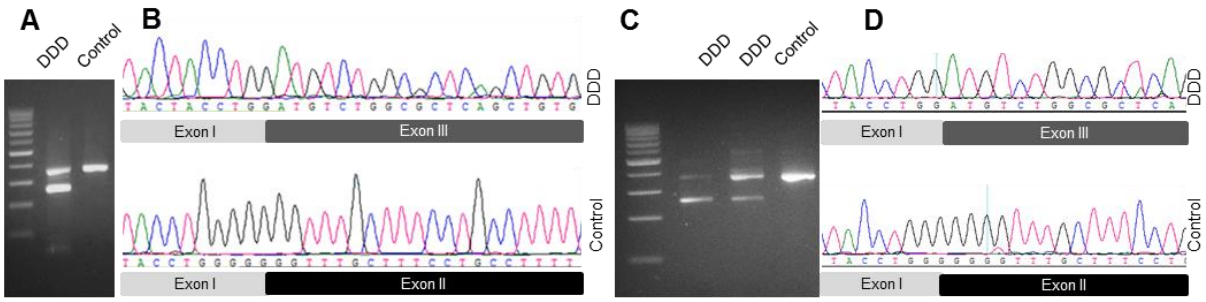
C. Downstream alternative acceptor site usage

ATGAACCTGGAGCGAGTGTCCAATGAGGAGAAATTGAACCTGTGCCGGAAGTACTACCTG**G**gtaaggcagatc…t
ggggtttgtttgtttgtttttctttctccctacGGGGGTTTGCTTTCCTGCCTTTTCTCTGGTTGGTCAACATCTTCTGGTTCTTC
CGAGAGGCCTTCCTTGTCCCAG**CC**TACACAGAACAGAGCCAAATCAAAGGCTgtgagtctagagcacagaggagggag
…ggctggtctagggaagtctggagagcagccggaggccaacccttccagcttctgtttcccatgacagATGTCTGGCGCTCAGCTGTG
GGCTTCCTCTTCTGGGTGATAGTGCTCACCTCCTGGATCACCATCTTCCAGATCTACCGGCCCCGCTGGGGTGC
CCTTGGGGACTACCTCTCCTTCACCATACCCCTGGGCACCCCCTGA

Supplemental Figure 4. *In silico* analysis of the splice site variant c.62-1G>C by Human Splice Finder (HSF) (3)

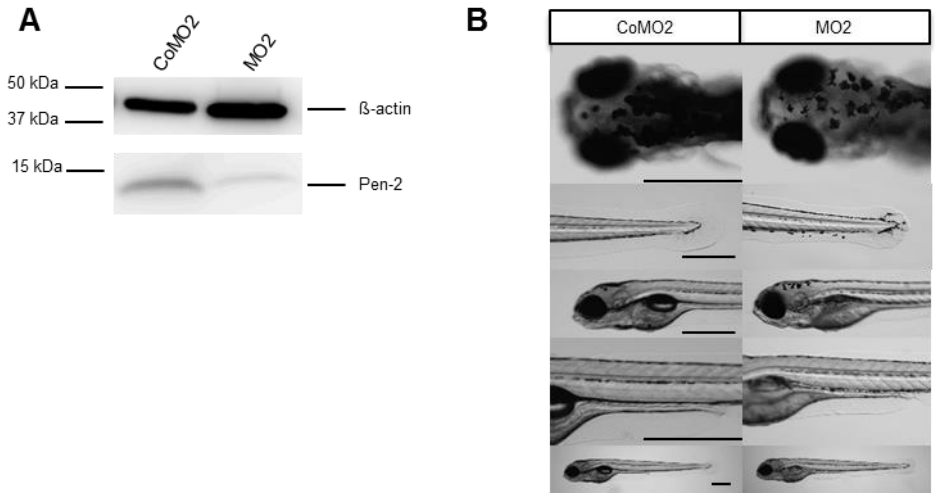
Exonic and intronic sequences are defined based on the wild type splice site usage and depicted with upper and lower cases. All three coding exons and their flanking introns are shown. Depicted with points are the intronic sequences, which are not included in the scheme. The codons are marked by alternating grey shading and unshaded units. (A) The wild type donor site (GT) of exon 1 and the wild type acceptor (AG) site of exon 2 are shaded in yellow. The HSF analysis calculated a consensus value of 98.15 for the wild type acceptor site of exon 2. Following the substitution c.62-1G>C (depicted with red in B and C), the wild type acceptor site is

broken with the consensus value reducing to 60.21. (B) One of the possible consequences of disruption of the acceptor splice site of exon 2 is exon skipping. The acceptor splice site of the following exon 3 is shaded in blue. Skipping of complete exon 2 results in an in-frame deletion of 35 amino acids (aa 21-55) encoded by the underlined nucleotides. The glycine residue at position 21 of the wild type protein is substituted by an asparagine (codon given in bold). (C) *In silico* splice site analysis revealed an alternative splice site (shaded with green) downstream of exon 2 which is assigned a consensus value of 87.66 by HSF. In case it is used, 29 aa (aa 21-49; underlined codons) are skipped in -frame. The glycine residue at position 21 of the wild type protein is substituted by an alanine (codon given in bold).



Supplemental Figure 5. Analysis of the splice site mutation c.62-1G>C and g.1412T>C

(A and C) Gel electrophoresis showing the wild type (WT) (362 bp) and the mutated (257 bp) transcripts. (B and D) Sequencing revealed skipping of the second exon of *PSENEN* as the consequence of the splice site mutation identified in the affected Indian individuals.



Supplemental Figure 6. Knockdown of *PSENEN* homolog *psenen* in zebrafish using MO2/CoMO2 confirmed pigmentation abnormalities

(A) Adequate knockdown of pen-2 by MO2 was confirmed by immunoblotting. (B) Representative zebrafish larvae phenotype observation at 96 hpf. Scale bar represent 500 µm.

Supplemental References

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