Homozygous *CDH2* variant causes hypopituitarism without neurological disorders.

Supplementary data

Methods

Table S1. Intronic primers of the *CDH2* gene.

Exon	GC Quantity	Fragment Length (bp)	Primer	Sequence (5'-3')
1	55	493	Forward	gcccgctatttgtcatcagc
	60		Reverse	gaaaggcgcgagagacctac
2	45.5	412	Forward	acccttgccctctacattcta
	44		Reverse	agtccctacagtctcatcatagtga
3	58.9	414	Forward	tcctttaacctaagcaggatataggt
	58		Reverse	atgtggtctgaagcaaagca
4	60	405	Forward	actgtgattcctatgctttcagggt
	59.2		Reverse	agaggctttctacaacactacaga
5	60.1	384	Forward	gtgattttctggggagagatggt
	58.2		Reverse	tcagtattgaggctttcagactg
6	58.2	419	Forward	tgggaagaaccgcttttgtt
	59.4		Reverse	ccttccttcctttcccaaaagcat
7	57.3	823	Forward	aggtctaaaatgaacctttttggaa
	58.9		Reverse	tgtgtgcaaattatttggctgtg
8	59.5	383	Forward	tgaatgatgccatgctctcct
	60.1		Reverse	tgtccgagttagcagccatg
9	59.9	369	Forward	aaaatcggacggtgtggtca
	57.9		Reverse	acgtcctaagtttccattgca
10	57.6	448	Forward	ttggaaatggagttgatagaggaa
	57.2		Reverse	tttgattgcaatgaaaatttctactca
11	58.2	399	Forward	tgtccagccatcatctacaga
	57.4		Reverse	aaaagctggacctcggaatt
12	60	403	Forward	aagggccagaaggttagaagc
	59.5		Reverse	attaacttttcatgccaggcttca
13	47	485	Forward	tgcagcatagttgtaatccgc
	55		Reverse	ggcgaggaaatgctgaaagg
14	60	406	Forward	agacgcatttcaggccttca
	59.7		Reverse	tcaacacacataggaaacaatttacca

Alternative *CDH2* splicing checked by cDNA amplification.

Table S2. PCR protocol for *CDH2* cDNA amplification**.**

Component	Volume
cDNA (Template DNA)	Variable
Forward Primer (10pmol/µL)	1 µL
Reverse Primer (10pmol/µL)	1 µL
5x Green GoTaq^{R} Reaction Buffer	5 µL
PCR Nucleotide Mix, 25 mM each	0.5 µL
GoTaq^{R} DNA Polymerase (5µ/µL)	0.3 µL
Betaine (Sigma Aldrich), 5M	2 µL
Nuclease-Free Water	Variable
Total Volume	25 µL

Table S3. Thermal cycling conditions for cDNA amplification by PCR.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	5'	1 Cycle
Denaturation	95 °C	30''	
Annealing	62 °C	30''	35 Cycles
Extension	72 °C	2'30''	
Final Extension	72 °C	10'	1 Cycle
Soak	4 °C	Indefinite	1 Cycle

Cell culture and generation of stable cell lines expressing human *CDH2*

The 2,721 bp open reading frame of *CDH2* (NM_001792.4) was cloned between the HindIII and BamHI sites of pcDNA3.1(+)-C-DYK (Genscript). Site directed mutagenesis was performed to create variants including the p.A77M negative control (c.229_231delinsATG; corresponding to mouse *Cdh2* p.A78M) (11) and p.V289I (c.865G>A, the patient variant) (Genscript). Clones were confirmed by Sanger sequencing of the open reading frame.

1.25E5 L1 cells were transfected with 2 µg of wild type, A77M or V289I *CDH2* plasmids in p60 dishes with Fugene6 according to the manufacturer's protocol (Promega). To establish stable cell lines expressing *CDH2*, we took advantage of the neomycin resistance gene in the pcDNA3.1 plasmid backbone and carried out drug-selection in media containing 1000 µg/ml G418 (Thermo-Fisher # 10131035). Ten days after the beginning of drug selection, cells were passaged, expanded and multiple aliquots frozen in regular media containing 8% dimethylsulfoxide (Sigma #D2650). All further experiments were executed using these aliquots. They were repeated twice.

Assessment of *CDH2* expression with quantitative PCR and immunocytochemistry

A confluent p60 dish of native L1 cells (untransfected), or stable cell L1 lines expressing *CDH2* wild type, A77M, or V289I was was washed with 1X phosphate buffered saline (PBS, pH 7.4, Thermo Fisher #10010-023), dissociated with 0.05 % trypsin-EDTA (ethylenediaminetetraacetic acid) (Thermo Fisher # 25300-062) and pelleted at 1,200 g, washed, and pelleted again. RNA was extracted (RNAqueous-4PCR Total RNA Isolation Kit, Thermo-Fisher #AM1914), DNase treated, and reverse transcribed with oligo(dT)12-18 primers into cDNA (SuperScript First-Strand Synthesis System for RT-PCR, Thermo Fisher # 11904018). Quantity and quality were assessed with a Nanodrop 2000 (Thermo Fisher). 50 ng cDNA, 1 µl TaqMan-probe (*Gapdh* Rodent Control #4308313, *Cdh2:* Mm01162497_m1, *CDH2*: Hs00983056_m1), and TaqMan Universal PCR Master Mix was used in a 20 µl quantitative PCR reaction performed in triplicate in an ABI Real-Time PCR 7500 with default thermocycling parameters (Thermo Fisher). Comparative threshold (C_T) values were plotted using Prism 7.0 (GraphPad).

For analysis of protein expression, 5E4 native L1 cells (untransfected), or stable cell lines expressing *CDH2* wild type, A77M, or V289I were plated onto glass coverslips (Corning #2870-18) in 6-well cluster plates (Corning Costar #3516) and incubated overnight. Cells were washed with 1X PBS, fixed for 20 minutes with 3% paraformaldehyde (Thermo Fisher F79-500). After a wash with 1X PBS, background was blocked for 20 minutes with 3% hydrogen peroxide (Sigma #216763) and 50% methanol (Thermo Fisher #A454-4), followed by 1 hour with biotin (Avidin Biotin Blocking kit, Vector Biolabs SP-2001) and 5% normal donkey serum (Jackson ImmunoResearch #017-000-001). Cells were incubated with 1:200 dilution of anti-cadherin-2 antibody (polyclonal, raised in rabbit, Abcam #ab12221) overnight in a

humidified chamber. For each cell line, two wells were incubated without primary antibody. The following day cells were washed with 1X PBS and a 1:200 dilution of anti-rabbit-biotin secondary antibody (Jackson ImmunoResearch #711-066-152) was applied for 1 hour. The biotin signal was developed using the TSA Fluorescein System (Perkin Elmer #NEL701001KT) according to the supplied protocol. Nuclei were co-stained with diamidino-2-phenylindole (DAPI, Thermo Fisher #62248) for 5 minutes. Coverslips were mounted onto standard microscope slides and imaged with Leica DMRB microscope, DFC310 FX camera, EL6000 light source and LAS v4.3 software (Leica).

The images taken were further analyzed with the FIJI basic pipeline for fluorescence analysis, we split the RGB channels to analyze the one with the *CDH2* marker, eliminated the noise background (50 pixels), set the threshold to select only the stained area, and measured the mean gray intensity for each point. We used the mean grey intensity of each point of the picture to calculate the mean gray intensity of the sample.

Cell aggregation with L1 fibroblast stably expressing wild type, p.A77M and p.V289I human *CDH2*

The fluorescent lipophilic stains, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ("DiI", "red", Thermo Fisher #D3911) or green with 3,3′-dioctadecyloxacarbocyanine perchlorate ("DiO", "green", Sigma #D4292), were dissolved as previously described[18]. 5E5 native L1 cells or stable cell lines expressing *CDH2* wild type, A77M, or V289I were plated into two standard 60 mm tissue culture treated plastic dishes (Corning #353004) in the afternoon. In the evening either the red or green stain was added as a 1:50 dilution. The next morning confluent cells were carefully rinsed twice with 2 ml per dish of a calcium wash solution (CWS) (1 mM calcium chloride, filter sterilized, Sigma C7902, in Hank's Balanced Salt Solution, HBSS, Thermo Fisher #14175). A homogenous cell suspension was prepared from each dish by treating with 4 ml of freshly diluted 0.01 % trypsin (Thermo Fisher #15050-065), 0.01 mM HEPES (Thermo Fisher #15630), and 1 mM calcium chloride in HBSS (TH-CWS) in a 37 ºC incubator with shaking at 100 revolutions per minute (rpm) for 20 minutes. Meanwhile, 60 mm glass dishes (Corning #3160-60) were washed with 70% ethanol, air-dried in the tissue culture hood, rinsed twice with CWS and loaded with 2 ml TH-CWS. From the 4 ml cell suspensions, 1 ml

of the red-labeled and 1 ml of the green-labeled cells of the same genotype were combined with 2 ml TH-CWS in one glass dish and immediately shaken in a 37 ºC incubator for 120 minutes at 80 rpm. Aggregates were fixed with the addition of 1 ml 4% glutaraldehyde (Sigma #G6257) in HBSS, and dishes were poured into 60 mm plastic dishes for imaging.

Five representative areas of each dish were imaged using an Olympus IX71 microscope, XM10 camera, Exfo X-Cite Series 120 light source, and Cellsens Standard software 1.5, build 9164. Cells and aggregates were counted manually using ImageJ cell counter plugin (NIH). The percent aggregation was calculated as ($N_{0\ min} - N_{120\ min}$) / $N_{0\ min}$, where $N_{0\ min}$ is the number of cells in the image at the outset, and $N_{120\ min}$ is the number of aggregates in the image at 120 minutes[18]. The number of cells forming each aggregate was also quantified. Results were graphed using Microsoft Excel 2016 (Microsoft). Variances within and between groups were tested with Shapiro-Wilk and Levene tests, and groups were compared either with one-way ANOVA and Scheffe post-hoc test or Mann-Whitney U-test using SPSS Statistics 25 (IBM). Level of significance was set to $p < 0.05$. Composite images were compiled with Adobe Photoshop and Illustrator CC 2018.

Beta catenin interaction assay

When cell cultures achieved 80-90% of confluence, they were passed with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, EUA) and plated in 24-wells at a concentration of $1x10^5$ cells/µL. The plasmid with the V289I allelic variant was constructed within the *CDH2* coding sequence (Origene, Rockville, MD, USA) using using QuikChange® XL Site-Directed Mutagenesis (Agilent Technologies), with primers R 5'-gtcagcatcaattgctgttatggtcatcacatatgttccag-3', F 5'-ctggaacatatgtgatgaccataacagcaattgatgctgac-3', according to the manufacturer's protocol.

Maintenance of Zebrafish (*Danio rerio*)

Use of zebrafish in this study was approved by the Medicine school of University of Sao Paulo

Committee on Laboratory Animals (Protocols 1675/2021)

All animals were kept in 3.5 L aquariums (Altamar /Brazil), with a distribution of 15 adult fish in each one. Animals are kept under controlled climate conditions, pH ~ 7.5; conductivity from 500 to 700µs; with system temperature of 28ºC+/-1. The

photoperiod will be 14 hours light and 10 hours dark. The animals are kept in the Central Animal Facility of the Faculty of Medicine at USP and fed three times a day with commercial Gemma micro feed (Skretting a nutreco company ®) in suitable sizes according to the age of the animal, which will vary from 75 to 300 microns.

Zebrafish reproduction

For zebrafish reproduction, the animals were separated, three males and three females, in a fertilization tank with a divider, the night before collection. The next morning, with the room light on, the partition between the animals was removed and after 10 to 15 minutes the eggs were collected, the injection was performed immediately after collection. After the injection, embryos were placed into a petri dish (90mm) with embryonic medium (50X solution: 14.69g NaCl , 0.63g KCl , 2.43g CaCl2.7H2O, 4.07g MgSO4.7H2O in 1L osmosis water). Petri dishes were placed in an oven at 28°C. During the period of five days, the plates were cleaned twice a day, with the removal and quantification of non-viable embryos.

Genomic editing by CRISPR/Cas9

The DNA template (Table S3) that gave rise to the guide RNAs was designed using the CRISPRscan.org platform, according to the protocol by Charles Vejnar et al. (Charles E. Vejnar, 2018).

Table S4. DNA template design for production of guide RNA for *cdh2* gene on Zebrafish.

Gene	Exon	Direction	Oligo (5'-3')
CDH2	3	Forward	taatacgactcactataGGCTGCCATAACTGTCCCAT
CDH2	3	Reverse	ttctagctctaaaacATGGGACAGTTATGGCAGCC
CDH2	3	Forward	taatacgactcactataGGGGACTGTGGCAGAGGTTC
CDH2	3	Reverse	ttctagctctaaaacGAACCTCTGCCACAGTCCCC
CDH2	3	Forward	taatacgactcactataGGGGATGAGGGTGCCAAACC
CDH2	3	Reverse	ttctagctctaaaacGGTTTGGCACCCTCATCCCC

***In vitro* transcription for guide RNA production**

In vitro transcription of sgRNA was performed according to the kit manual (GeneArt Precision gRNA Synthesis Kit, Thermofisher Scientific). The first step was the assembly of the gRNA DNA template:

a. 0.3 µM oligonucleotide working solution was prepared by diluting the 10 µM primer stock solution in nuclease -free water.
b. The Mix was made to proceed with the PCR (Table 3 and 4).
c. The product was incubated at 37°C for 4 hours in a thermocycler in 0.2ml tubes.
d. After transcription, 1 µL of DNase I was added to the MIX and then incubated at 37°C for 15 minutes.
e. RNA purification was performed according to the kit.
f. Agarose gel run was performed to verify RNA integrity: 0.5 µL of RNA produced in 10 µL of RNASE-free water adding 7 µL of 2X RNA loading dye Solution.
g. RNA quantification was then performed in a spectrophotometer (Bio Photometer Eppendorf): by diluting sgRNA 1:10 in RNASE-free water.
h. We proceed to Microinjection

Table S5. PCR protocol for *cdh2* sgRNA amplification**.**

Component	Volume
Phusion™ High-Fidelity PCR Master Mix (2X)	12.5 µL
Tracr Fragment + T7 Primer Mix	1 µL
0.3 uM of forward and reverse primers	1 µL
Nuclease Free Water	10.5 µL

Table S6. Thermal cycling conditions to build Zebrafish *cdh2* sgRNA by PCR.

Step	Temperature	Time	Number of Cycles
Initial			
Denaturation	98 °C	10''	1 Cycle
Denaturition	98 °C	5''	32 Cycle
Annealing	55 °C	15''	
Final Extension	72 °C	1'	1 Cycle
Soak	4 °C	Indefinite	1 Cycle

Table S7. *In vitro* transcription reaction (IVT) protocol for sgRNA.

Component	Volume
NTP Mix (100 mM each of ATP, GTP, CTP, UTP)	8 µL
gRNA DNA (PCR product)	6 µL
5X TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

Microinjection

sgRNA production and quantification in nanodrop , the mix for injection was prepared . For knockout, 100ng/ ul Cas9, 80ng/ ul sgRNA in a total of 5 ul completing with RNAse -free water adapted from the protocol by Charles E. Vejnar et al (Charles E. Vejnar, 2018). The injection needles were made with a glass capillary (Borosilicate glass, Sutter instrument , USA) in a *puller into micropipettes* (Sutter instrument , USA) which were filled with 10 µL of MIX using a microloader (Eppendorf). The needle was then placed in the holder connected to a pneumatic microinjection pump (Harvard device, USA). Injections were made at 50X magnification under a stereomicroscope (SMZ 745, Nikon, Germany). The injection volume was calibrated using a micrometric ruler (Bresser 1/10 mm) (Edmunds JS, 2000). According to the sphere volume formula ($V = 1 / 6\pi d3$), a sphere diameter of 1 bar corresponds to an

injection volume of 1 nL . 1nL of the respective mix was injected into the cell of the embryo at the unicellular stage.

Genomic extraction

24 hours after the knockout injection, genomic DNA was extracted from the embryos to verify the efficiency of the knockout technique (from 5 to 10 embryos). Briefly, animals with 1 day post fertilization (dpf) were placed on ice for euthanasia and then placed in a 0.2ml microtube containing 20 µl of 20 mM NaOH . These samples were incubated at 95°C for 25 minutes, cooled to 4°C, and neutralized with 4 µl of 1 M Tris- HCl (pH 8.0) and followed for PCR amplification and sequencing.

Amplification of the target region for genotyping

Primers were designed to flank the cleavage site of *cdh2* exon 3 (Table S7).

Table S8. Primers for PCR amplification of Zebrafish *cdh2* exon 3.

Gene	Exon	Direction	Oligo (5'-3')	Fragment Length (bp)
cdh2	3	Forward	CCCTTGTGGAGGAGTGGAAT	679
cdh2	3	Reverse	ATATGCTTGCTGGGCAACTT	679

Table S9. PCR protocol for *cdh2* exon3 amplification.

Component	Volume
DNA	2 µL
Platinum™ Taq DNA Polymerase	0.2 µL
$MgCl_2$	1 µL
5x Platinum II PCR Buffer	2 µL
10 mM dTNP mix	0.4 µL
Forward Primer (20mM)	0.4 µL
Reverse Primer (20mM)	0.4 µL
Nuclease-Free Water	18.6 µL
Total Volume	25 µL

Table S10. Thermal cycler program for *cdh2* gene PCR.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	94 °C	2'	1 Cycle
Denaturation	94 °C	30''	35 Cycles
Annealing	72 °C	30''	
Final Extension	1 °C	30''	1 Cycle
Soak	4 °C	Indefinite	1 Cycle

Sequencing for variant confirmation

2.5 µL of the amplified PCR product was purified with 1 µL of ExoSAP -IT enzyme (TermoFisher Scientific) and sequenced in the ABI PRISM 3100 Genetic equipment analyzer (TermoFisher Scientific). The sequences obtained were analyzed using the Sequencher software (Gene Codes Corporation, Michigan, USA), comparing with the reference gene sequence extracted from the Ensembl database

Table S11- *CDH2* Allelic variants found in the whole cohort. HT: heterozygous. HM: homozygous. NF: Not found.

Variant	Number of Patients		AbraOM		1000 Genomes		gnomAD v2.1.1		gnomAD v3		ACMG VERDICT
	HT	HM	HT	HM	HT	HM	Allele Frequency	HM	Allele Frequency	HM	
c.-62 T>G	48	9	0.164992	18	0.213 (534)	0.026 (64)	0.00017	462	0.1818	2334	Benign
c.-65T>G	2	0	0.0041	NF	0.005 (13)	NF	0.0009	0	0.00005808	0	VUS
c.-38_-36DUPGCC	0	1	NF	NF	NF	NF	0.00423	317	0.000001993	1282	Benign
c.-41_-36DELGCCGCC	0	1	NF	NF	NF	NF	0.03371	107	NF	NF	VUS
c.333C>G	7	0	0.032	NF	0.085 (214)	0.004 (9)	0.04503	358	0.426	145	Benign
c.352G>A	7	0	0.032	NF	0.085 (214)	0.004 (9)	0.04493	359	0.0424	143	Benign
c.696G>T	2	0	0.0016	NF	0.011 (28)	0 3(1)	0.0018	9	0.0053	9	Likely Benign
c.865G>A	1	1	0.0016	NF	0.003 7(95)	NF	0.00371	0	NF	NF	Likely Benign
c.1202C>A	1	0	NF	NF	NF	NF	NF	NF	NF	NF	Likely Pathogenic
c.1430_1431DELCCINSTG	1	0	NF	NF	NF	NF	NF	NF	NF	NF	Likely Pathogenic
c.2388G>A	1	0	NF	NF	0.001 (3)	0.999 (2501)	0.00005	0	0.0001	0	Likely Benign
c.2448C>T	50	12	0.362069	85	0.423 (1060)	0.116 (290)	0.3359	16924	NF	NF	Benign

c.2534A>G	1	0	0.024631	NF	0.04 (100)	0.001 (3)	0.02483	123	0.0199	41	VUS	

Table S12- Number of animals injected with cdh2 sgRNA and survival rate. hpi: Hours post injection.

Groups	Total Injected	Live Animals				Survival rate	Mortality rate
		24 hpi	48 hpi	72 hpi	96 hpi		
C1	131	108	99	84	76	58%	42%
C2	71	39	34	30	20	28%	72%
C3	150	114	110	96	38	25%	75%

Figure labels.

Figure S1.

CDH2 cDNA amplification of the patient reveals no difference from the positive control band.

Figure S2. Immunofluorescence analysis of L1 mouse fibroblasts transfected or not with *CDH2* variants shows no difference in protein levels.

Figure S3. Dual luciferase assay with HEK293 cells co-transfected with $CDH2^{V289I}$ plasmid reveals no effect in cadherin capture of β-catenin. In black is the luciferase internal control, which was used to normalize the data, WT results are expressed in white and $CDH2^{V289I}$ co-transfected cells are represented in pink.

Figure S4. *cdh2* Knockout in zebrafish causes morphological abnormalities (A-F). Red dashed circle: Microphthalmia; Red arrow: Tail curvature; Black arrow: Pericardium edema; Black arrowhead: Non formation of the tail/Somite malformation; Red asterisk: Yolk sac edema.

Figure Captions

Figure S1.

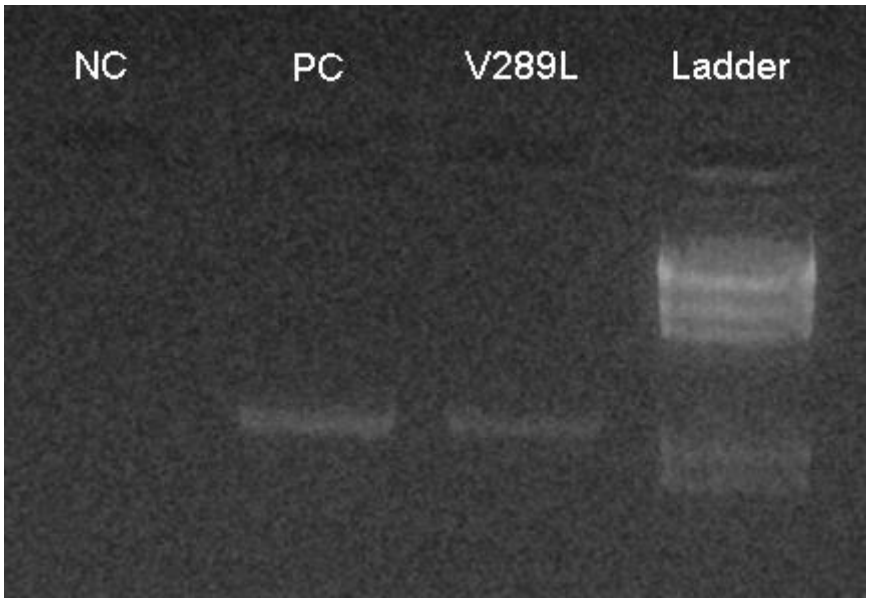


Figure S2.

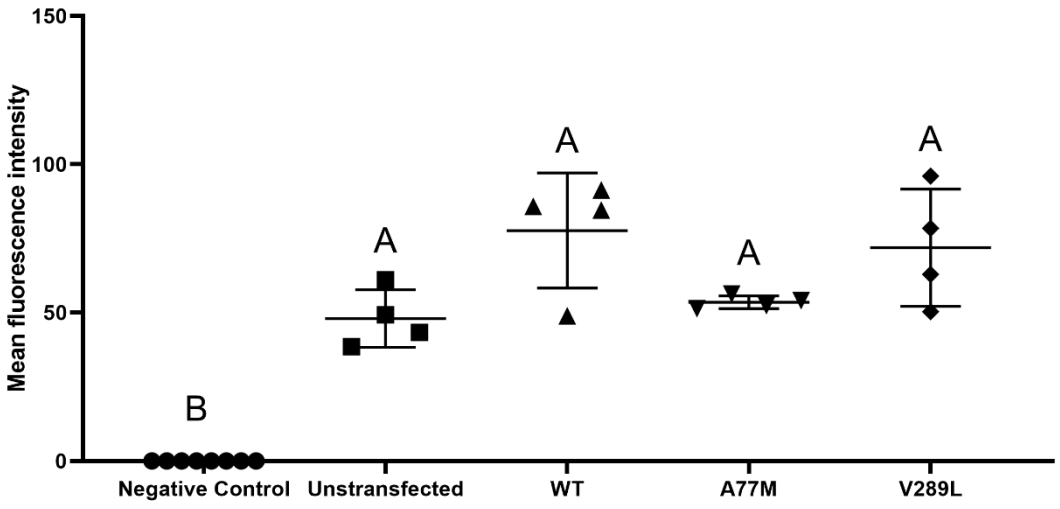


Figure S3.

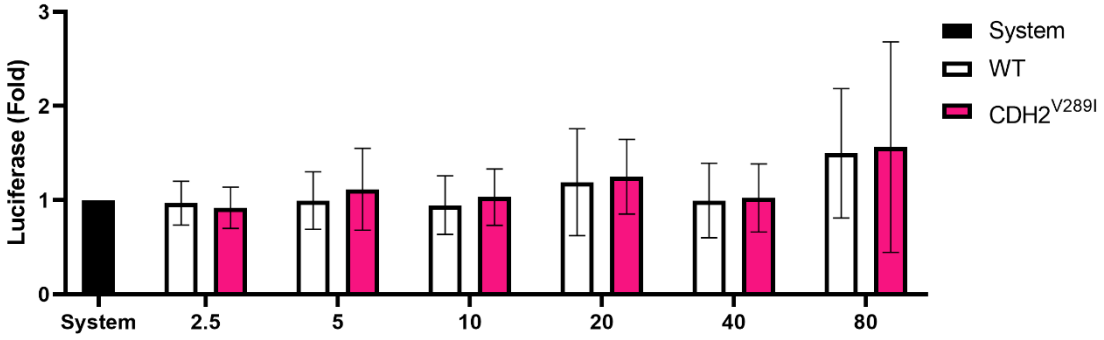
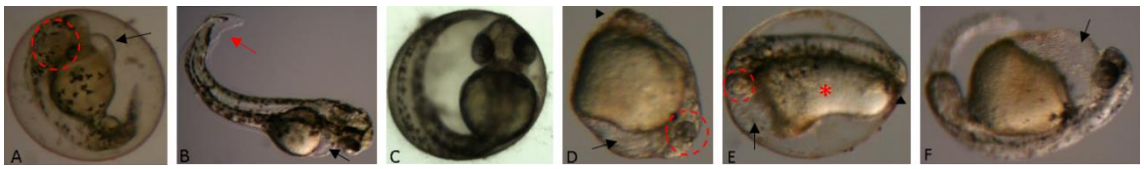

Luciferase (Fold)
3
2
1
0
System
2.5
5
10
20
40
80
System
WT
CDH2V289I

Figure S4.


A
B
C
D
E
F