A novel gain-of-function mutation of Piezo1 is functionally affirmed in red blood cells by high-throughput patch clamp

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A novel gain-of-function mutation of Piezo1 is functionally affirmed in red blood cells by high-throughput patch clamp

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Supplemental Material

Materials and Methods

Molecular analysis

The DNA sample of the patient was analysed on an NGS-targeted panel SureDesign software (Agilent Technologies, Santa Clara, USA), containing 40 genes associated with congenital haemolytic anaemia (Supplemental Table S1). Libraries were obtained by HaloPlexHS Target Enrichment System Kit and sequenced on a MiSeq platform (Illumina, San Diego, USA). Only the causative mutation R2110W in *PIEZO1* gene was detected in exon 44. Sanger sequencing was performed on an ABI310 DNA Analyser (Applied Biosystems, Foster City, CA, USA). The novel variant was considered pathogenetic according to the guidelines of the American College of Medical Genetics. Prediction was made according to the following algorithms: SIFT; PolyPhen-2; VarSome; MutationTaster; PredictSNP. MAF was based on the following databases: ExAc; 1000G.

Cell culture and harvesting

Neuro2A (N2A) mouse neuroblastoma cell line was kindly provided by Max Delbrück Centre (Berlin, Germany). Frozen stocks were thawed following the manufacturer's instructions, cultured for at least 4 days after thawing and used on the automated patch clamp systems 4 to 10 days after plating. Human red blood cells (RBCs) were freshly obtained from the fingertips of the experimenter (healthy volunteer) upon written informed consent and suspended in physiological Ca^{2+}-free external solution. *PIEZO1*-mutated (R2110W) and control blood samples were shipped overnight from Milan (Italy) to Munich (Germany) to enable recordings within 24h after blood withdrawal. Samples were shipped in heparin-coated tubes at 4°C.

Patch clamp solutions

For automated patch clamp of all cell types, internal solution contained (in mM): 10 KCl, 110 KF, 10 NaCl, 10 EGTA and 10 HEPES/KOH (pH 7.2). External recording solution contained in mM: 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Glucose and 10 HEPES/NaOH (pH 7.4). Yoda1 (10 µM; Sigma-Aldrich), $GdCl_3$ (30 µM; Sigma-Aldrich) and TRAM-34 (500 nM; Sigma-Aldrich) were prepared in external recording solution.

Automated patch clamp recordings

Whole-cell recordings were carried out on the SyncroPatch 384PE (Nanion Technologies, Munich, Germany). The SyncroPatch 384PE used in this work consists of an automated patch clamp module in combination with a 384-channel digital amplifier (Tecella, Foothill Ranch, CA), a CyBio FeliX (AnalytikJena, Jena, Germany) pipetting robot with a 384 pipettor arm and proprietary softwares for data acquisition (PatchControl 384, Nanion Technologies) and data analysis (DataControl 384, Nanion Technologies). All recordings were performed at room temperature using planar borosilicate glass patch clamp chips (Fertig et al., 2002) in a 384-microtiter-plate format with medium (3-5 MΩ for N2A cells) and ultra-high (9-12 MΩ for RBCs) resistances. Prior to the electrophysiological measurements, N2A cells were detached by accutase treatment (5-10 min), washed with culture medium and adjusted to a final density of $2x10^5$ cells/ml. Whole blood from fresh control, shipped control and Piezo1 patient samples was suspended in Ca^{2+}-free external solution to a final haematocrit of ~0.05% for fresh control and ~0.5% for shipped control and patient samples. Cell suspensions were kept in the dedicated cell reservoir at 10°C and used for no longer than 2 h. Currents were elicited using voltage-ramps from -100 to + 80 mV with 300 ms duration. Initial tests to investigate Piezo1 channels via mechanical stimulation, i.e. pressure steps or fluid shear stress, failed to activate the current in several attempts. We therefore assumed no or very little contribution of mechanical activation of Piezo1 in our recordings. Piezo1 channel investigation in both N2A and RBCs was performed using a Piezo1 chemical specific agonist, Yoda1 (Syeda et al., 2015) and $GdCl_3$ as a non-selective stretch-activated channel inhibitor. To investigate Gardos current contribution to the Yoda1-activated currents in RBCs, TRAM-34 (Gardos specific inhibitor) was applied along with the external washing solution and kept for the duration of the experiment. The resulting currents were tested with Yoda1 and blocked by $GdCl_3$.

References

Fertig N, Blick RH, Behrends, JC, Whole cell patch clamp recording performed on a planar glass chip. Biophysj. 2002. 82, 3056–3062.

Syeda R, Xu J, Dubin AE, Coste B, Mathur J, Huynh T, et al. Chemical activation of the mechanotransduction channel Piezo1. Elife. 2015;4.

Supplemental Table 1: Clinical and haematological data at the time of the study

	Patient R2110W	**Reference values**
Age (years)	43	
Transfusions	no	
Splenomegaly	no	
Hb (g/dL)	16.9	13.4-17.5
MCV (fL)	80.9	80-94
MCHC (g/dL)	39.1	31-37
Reticulocytes (x10^9/L)	193	20-100
RBCs morphology	7% stomatocytes	
Unconj. bilirubin (mg/dL)	0.66	<1
Serum ferritin (ng/mL)	546	30-400
AGLT	>900	>900
Pink test	7	11-33
NaCl osmotic fragility	decreased	
EMA binding test	normal	

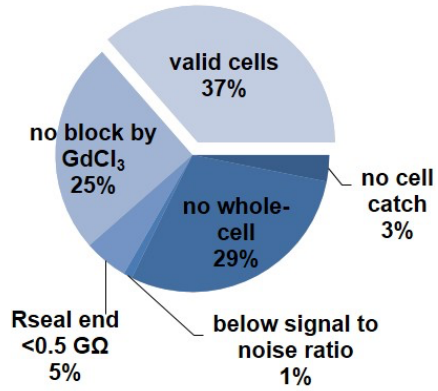
Supplemental Table 2: Genes associated with congenital haemolytic anemias investigated through a 40 genes-targeted Next Generation Sequencing panel.

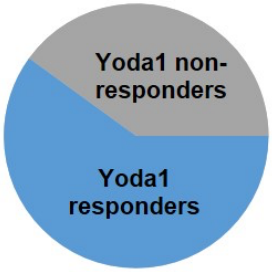
Gene	Ref. Sequence	Gene	Ref. Sequence
ABCB6	NM_005689	**GSS**	NM_000178
ABCG5	NM_022436	**HK1**	NM_033497
ABCG8	NM_022437	**KCNN4**	NM_002250
ALAS2	NM_001037967	**KIF23**	NM_138555.2
AK1	NM_000476	**KLF1**	NM_006563.3
ALDOA	NM_000034	**NT5C3A**	NM_016489.12
BPGM	NM_001293085	**PFKL**	NM_001002021
C15ORF41	NM_001130010	**PFKM**	NM_000289.5
CDAN1	NM_138477	**PGK1**	NM_000291.3
CYB5R3	NM_000398	**PGM1**	NM_001172819
ENO1	NM_001201483	**PKLR**	NM_000298.5
EPB41	NM_004437.3	**PIEZO1**	NM_001142864.2
EPB42	NM_000119.2	**RHAG**	NM_000324.2
G6PD	NM_000402	**SEC23B**	NM_006363.4
GATA1	NM_002049	**SLC2A1**	NM_006516
GCLC	NM_001498.3	**SLC4A1**	NM_000342.3
GCLM	NM_001308253	**SLC25A38**	NM_017875.2
GPI	NM_000175.3	**SPTA1**	NM_003126.2
GPX1	NM_000581.2	**SPTB**	NM_000347.5
GSR	NM_000637	**TPI1**	NM_000365.5

Supplemental Table 3: Quality Control (QC) filters implemented to determine Yoda1 responder and non-responder cells. Piezo1-activated N2A cells and RBCs were selected based on the listed criteria to divide the cells as Yoda1 responders and Yoda1 non-responders. Inf stands for infinite.

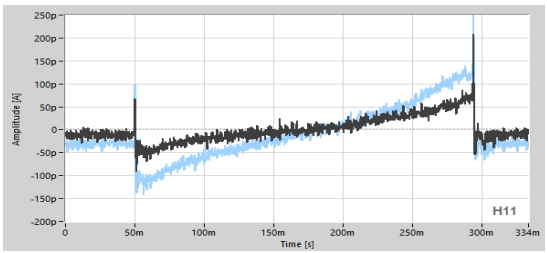
	Quality Control filters	N2A cells		RBCs	
		lower value	higher value	lower value	higher value
Seal resistance (MΩ)	**cell catch**	5	Inf	5	Inf
	whole-cell	50	Inf	50	Inf
	before compound addition	500	Inf	900	Inf
	end of the experiment	500	Inf	1500	Inf
Current (pA)	**inward current values**	-Inf	-20	-Inf	-20
	outward current values	16	Inf	16	Inf
	$GdCl_3$ block	20%	-	20%	-
	Yoda1 responders	20%	-	20%	-

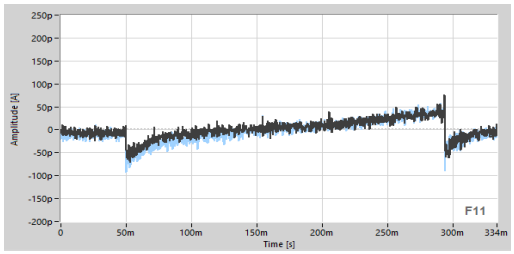
A

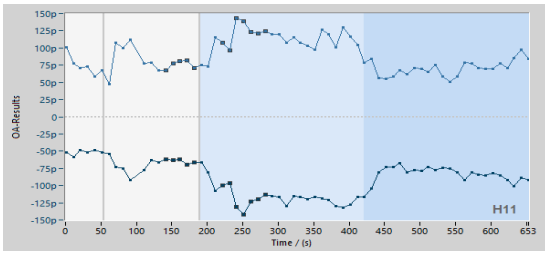

valid cells
37%
no block by
GdCl3
25%
no cell
catch
3%
no whole-
cell
29%
Rseal end
<0.5 GΩ
5%
below signal to
noise ratio
1%

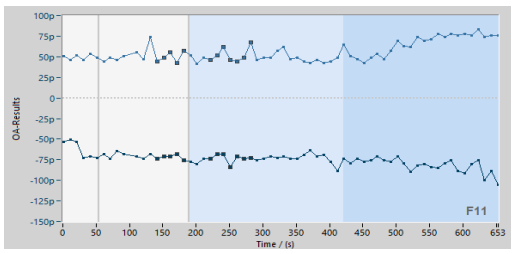

Yoda1 non-
responders
Yoda1
responders

B

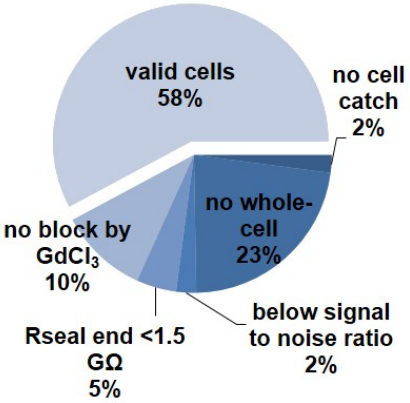

Amplitude [A]
Time [s]
H11

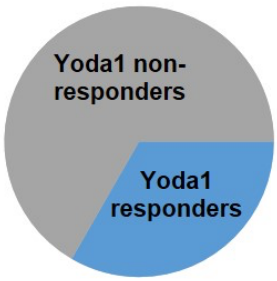

Amplitude [A]
Time [s]
F11


OA-Results
Time / (s)
H11

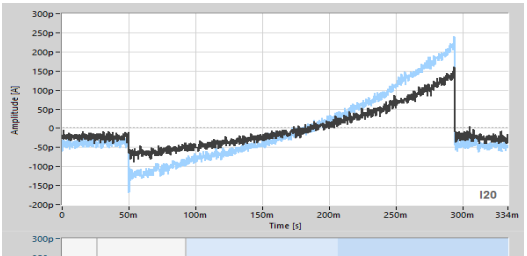

OA-Results
Time / (s)
F11

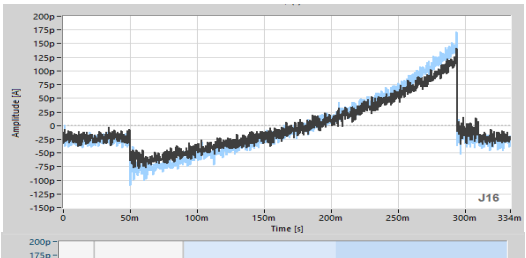
C

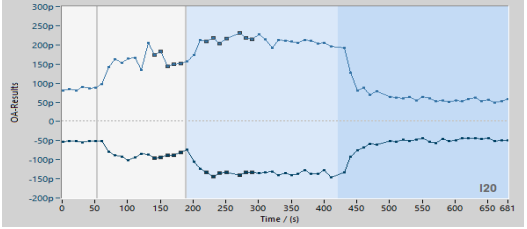

valid cells
58%
no cell
catch
2%
no whole-
cell
23%
no block by
GdCl3
10%
Rseal end <1.5
GΩ
5%
below signal
to noise ratio
2%

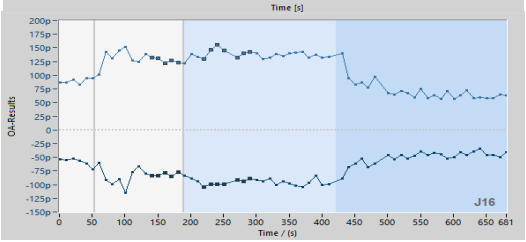

Yoda1 non-
responders
Yoda1
responders

D

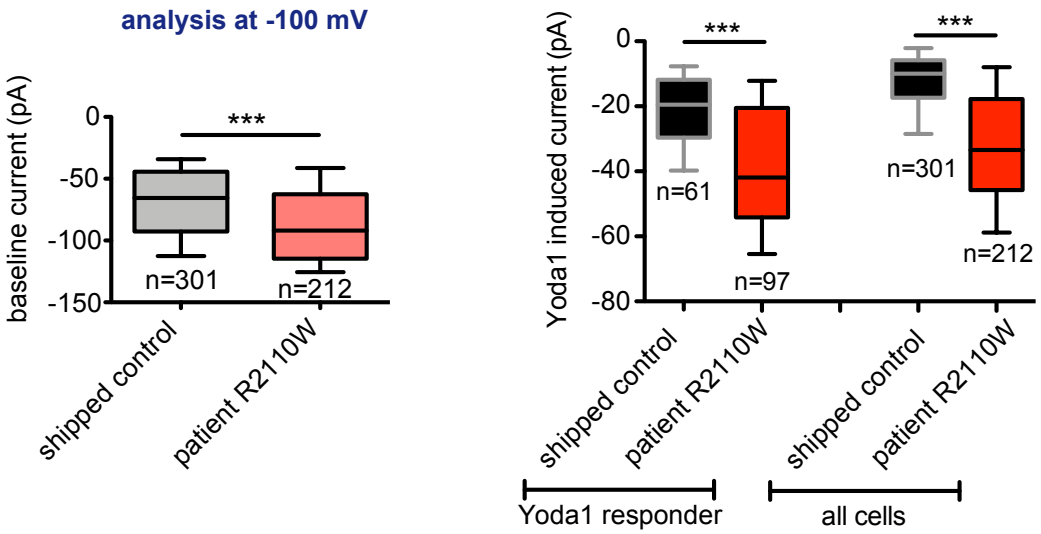

Amplitude [A]
Time [s]
I20


Amplitude [A]
Time [s]
J16


OA-Results
Time / (s)
I20


OA-Results
Time / (s)
J16

Supplemental Figure S1: Typical recording from Piezo1 endogenously expressed in N2A cells and in healthy human RBCs on the SyncroPatch 384PE. (A) Percentage of valid cells obtained following application of the QC filters listed in Supplemental Table 2 in one example NPC-384 chip. 140 out of 384 N2A cells (37%) passed the QC criteria and 85 cells (60% of the valid cells) were considered as Yoda1 responders. **(B)** Example of Yoda1 responder (left graph) and non-responder (right graph) N2A cells. Top: raw data trace of a single well recorded in external solution (black trace) and in the presence of 10 µM Yoda1 (light blue trace). Bottom: Online Analysis plot showing the positive and negative peak currents over time. Vertical lines indicate solution additions, white areas mark external (wash) solution, light blue marks 10 µM Yoda1, blue marks 30 µM $GdCl_3$. An average of the data points highlighted in the plot is used for the analysis. **(C)** Percentage of valid cells obtained following application of the QC filters in one example NPC-384 chip. 262 out of 384 RBCs (58%) passed the QC criteria and 74 cells (33% of the valid cells) were considered as Yoda1 responders. **(D)** Example of Yoda1 responder (left graph) and non-responder (right graph) RBCs. Top: raw data trace of a single well recorded in external solution (black trace) and in the presence of 10 µM Yoda1 (light blue trace). Bottom: Online Analysis plot showing the positive and negative peak currents over time. Vertical lines indicate solution additions, white areas mark external (wash) solution, light blue marks 10 µM Yoda1, blue marks 30 µM $GdCl_3$. An average of the data points highlighted in the plot is used for the analysis.



Supplemental Figure S2: Additional statistical analysis of recordings from cells with *PIEZO1* mutation (R2110W) cells compared to healthy human RBCs. The voltage protocol was the same as described in Figure 2A, except the holding potential was set to -30 mV. Because the current amplitudes did not show a Gaussian distribution they were presented as median and box plots (25% - 75%) with whiskers (10% -90%) in patient cells compared to controls. The numbers adjacent to the boxes refer to the numbers of cells measured. Here analysis of negative -100 mV membrane potential is shown exclusively to complement the analysis at positive membrane potentials depicted in Figure 3C. Only the cells passing the quality control filters (Supplemental Figure 3) were processed. In control condition, 61 out of 301 RBCs (20% of the valid cells) were considered as Yoda1 responders. In patient condition, 97 out of 212 (46% of the valid cells) were considered as Yoda1 responders. The baseline values (left) were already indicating a highly significant difference between patient and control. A difference in the Yoda1-induced current was evident when considering only the cells responding to Yoda1 and was sustained when all measured RBCs were included.