Multiple clinical forms of dehydrated hereditary stomatocytosis arise from mutations in *PIEZO1*

Supplemental Data Section comprises Material and Methods, 6 figures, their respective figure legends and a table.

Cell culture

Human HEK-293 cells (ATCC, Philadelphia, PA, USA) were maintained in DMEM medium (Sigma, Milano, Italy), supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies, Monza, Italy), 100 U/mL penicillin (Life Technologies, Monza, Italy), and 100 mg/mL streptomycin (Life Technologies, Monza, Italy), in a humidified 5% CO2 atmosphere at 37° C.

Exome capture and sequencing

Blood was obtained for genetic analysis from affected and unaffected family members of all analyzed families, and from healthy controls, with signed informed consent according to the Declaration of Helsinki. Blood collection was according to protocols approved by local university ethics committees. Genomic DNA was prepared from peripheral blood with the Wizard Genomic DNA purification kit (Promega, Milano, Italy). 5µg of DNA from two affected and two unaffected members of DHSt family Edinburgh was diluted in 700 µl of Nebulization buffer (Illumina, San Diego, CA) and sheared by nebulizers (Invitrogen, Carlsbad, CA) into fragments of 200–400 bp in length (Bioanalyzer 2100, Agilent, Santa Clara, CA). Sheared samples purified by QIAquick spin columns (Qiagen, Hilden, Germany) were processed for library preparation (Illumina protocol), omitting size-selection of adapter-ligated fragments prior to capture. After several cycles of PCR amplification, 500 ng of DNA from the resulting libraries was hybridized to the bait set from the SureSelect Human All Exon Kit (Agilent, Santa Clara, CA, USA) at 65°C for 24 h. Hybrid capture with streptavidin-coated Dynal magnetic beads (Invitrogen, Carlsbad, CA) was performed as manufacturer's protocol. Captured samples were further purified through Agencourt AMPure XP beads and subjected to PCR amplification. All samples at each step of library preparation were quantified by Bioanalyzer 2100 (Agilent). Individual sample libraries were NaOH-denatured and loaded onto one lane of an Illumina Flowcell v4. DNA clusters were generated through a one-step workflow on the Cluster Station using TruSeq PE Cluster Kit v5 (Illumina, San Diego, CA). A PhiX control library added to each sample at 1% volume served as internal control. Sequencing was performed on the Illumina Genome Analyzer IIx platform as paired-end 100-bp reads according to

the manufacturer's protocol. An exome capture was considered successful if >80% of the target regions were covered with a high quality genotype.

Directed DNA sequencing

PIEZO1 mutations were sought by direct sequencing. All exons and flanking splice junctions of the *PIEZO1* were PCR-amplified from 75ng genomic DNA in 25 µl with KAPA2G Robust HotStart ReadyMix 2X (KAPABYOSYSTEM, South Africa). Oligonucleotide primers were designed by the program Primer3 v.0.4.0 to avoid amplification of the homologous genes *PIEZO2/FAM38B*, *PIEZO1P1,* and *PIEZO1P2* (Ensembl Gene ID's ENSG00000154864, ENSG00000233686, ENSG00000237121). Primer sequences are available on request (achille.iolascon@unina.it).

Total RNA from whole blood of patient SF was isolated with the RNEasy Mini-Kit (Qiagen, Milan, Italy) and reverse transcribed (Retroscript, Ambion, Monza, Italy). cDNA encoding segments of *PIEZO1* was PCR-amplified using primers available on request (salper@bidmc.harvard.edu). Integrity of PCR products was checked by agarose gel electrophoresis. cDNA fragments purified from agarose gel were directly sequenced using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Branchburg, NJ). Missense substitution mutations in PIEZO1 were evaluated with PolyPhen-2, SIFT and Panther.

Evolutionary conservation analysis of PIEZO1 protein

Human PIEZO1 protein orthologs from other species (UniProtKB 15.0) were subjected to multiple sequence alignment (NCBI BLASTP 2.0.12). The aligned PIEZO1 sequences included *Homo sapiens* (Q92508), *Macaca mulatta* (H9F7J7), *Mus musculus* (E2JF22), *Rattus norvegicus* (Q0KL00), *Xenopus tropicalis* (F6SPB7 or XP002933721) and *Danio rerio* (XP696355).

Collection and immunohistochemistry analysis of mouse and human embryos

We carried out immunohistochemistry analyses on C57BL6/J mouse embryonic tissues at E12,5, E15,5 and P0, and on fetal human tissues harvested at 17 and 19 weeks of gestation. The human tissues were obtained according to protocols approved by local university ethics committees, after obtaining signed informed consent according to the Declaration of Helsinki.

Serial sections of 4 microns thickness were cut from paraffin blocks of embryonic mouse and of fetal human tissue, mounted on acid-cleaned glass slides, and heated at 55°C for 60 min. Slides were de-paraffinized and rehydrated; endogenous peroxidase activity was inhibited by treatment

with 3% H_2O_2 in methanol (20 min at room temperature). To reduce nonspecific background staining, slides were pre-incubated with 5% goat serum (20 min at room temperature). Slides were then incubated with polyclonal anti-PIEZO1 (Proteintech 15939-1-AP, 1:300) in a moist chamber for 1 h at room temperature, then developed with avidin-biotin-peroxidase complex (Vector Laboratories, Peterborough, United Kingdom) and diaminobenzidine, weakly counterstained with Harris' hematoxylin, and mounted. Specificity of the immunohistochemical signal was confirmed by the absence of staining in the absence of primary antibody.

Isolation and erythroid differentiation of CD34+ hematopoietic progenitor cells

Peripheral blood was collected from four healthy volunteers (with consent according to Declaration of Helsinki) and processed within 24 h. Mononuclear cells were isolated using Ficoll-Hypaque (1.077-0.001 kg/L; Sigma-Aldrich, Milan, Italy). The remaining red blood cells were lysed by resuspension of the cell pellets in lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). CD34+ cells were isolated by positive selection using the miniMACS immunomagnetic isolation system (Miltenyi Biotec, Glodbach, Germany), with ~ 95% yield. These CD34+ cells were cultured at 10^5 cells/mL in α-MEM (GIBCO, Grand Island, NY) supplemented with 30% FBS (GIBCO) and with 2 mM L-glutamine, 1 μM hydrocortisone, 0.1 mM mercaptoethanol, 1% bovine serum albumin in deionized water, 10 U/mL Penicillin/Streptomycin, 25 μg/mL gentamycin, (Sigma, St. Louis, MO). To induce erythroid differentiation of CD34+ cells, 10 U/mL recombinant human erythropoietin (rHuepo, Janssen-Cilag, Milan) was added to complete α-MEM medium, and cells were incubated 14 days at 37 °C in a 5% CO2 atmosphere. Cell samples were collected on days 0, 7 and 14 for determination of cell number and viability (Trypan Blue exclusion). Erythroid differentiation was assessed by expression of transferrin receptor-1 (CD71) and glycophorin A (CD 235A) as detected by FACS.

RNA isolation, cDNA preparation and quantitative qRT-PCR

Total RNA was extracted from cell lines, and tissues using Trizol reagent (Life Technologies). Synthesis of cDNA from total RNA (2 μg) was performed using Super Script II First Strand kits (Life Technologies). Quantitative RT-PCR (qRT- PCR) was performed using the SYBR-green method, following standard protocols with an Applied Biosystems ABI PRISM 7900HT Sequence Detection system. Relative gene expression was calculated using the 2(-ΔCt) method, where ΔCt indicates the differences in the mean Ct between selected genes and the internal control (GAPDH).

qRT-PCR primers for each gene were designed with Primer Express 2.0 (Life Technologies) to avoid amplification of the homologous gene products *PIEZO2, PIEZO1P1 and PIEZO1P2* (Primer sequences are available upon request).

Immunoblotting

Crude tissue lysates from newborn mouse lung, liver, spleen, and bone marrow were freshly prepared and solubilized in SDS-load buffer. Tissue and total cell lysates were run on SDS-polyacrylamide gels, then blotted onto polyvinylidene difluoride (PVDF) membranes (BioRad, Milan). PVDF membranes were incubated with the following antibodies: polyclonal rabbit anti-PIEZO1 (1:500; Proteintech 15939-1-AP; validated previously by Eisenhoffer et al.).[1] anti-GAPDH antibody (1:1000; Cell Signaling Technology, Danvers, MA) used as loading control, and HRP-conjugated anti-rabbit Ig (1:5000) (GE Healthcare, UK). Membranes were incubated in enhanced chemiluminescence (ECL) substrate (Supersignal West Pico Chemiluminescent Substrate Kit, ThermoScientific, Miami, FL). Labeled band visualization and densitometric analysis were performed with the BioRad Chemidoc using Quantity One software (BioRad).

Red blood cell membrane preparation

Peripheral blood from healthy controls was lysed by 3 cycles of freezing and thawing in 5mM KH_2PO_4 containing a protease inhibitor cocktail (Calbiochem, Merck KGaA, Darmstadt, Germany, set III, animal-free). The membrane fraction was sedimented by centrifugation of the lysate at 36,000 g for 20 min at 4°C. Membrane extracts were then loaded on SDS-polyacrylamide gels as previously described for total cell lysates. Mouse red cell membranes were prepared from 8 mL of blood collected from 30 healthy C57BL/6 mice, as described above for human red cells.

Red blood cell fractionation for immunofluorescence analysis

Human peripheral blood from control subjects was fractionated at room temperature by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, AXIS-SHIELD PoCAs, Oslo). RBCs below the first Ficoll-Hypaque interface were collected for preparation of blood smears and subsequent immunofluorescence analysis.

Immunofluorescence analysis

$2x10^6$ CD34+ cells (induced to erythroid differentiation as previously described) were washed in 1

mL PBS, centrifuged 5 min at 1000 g and fixed for 20 minutes with 4% (w/v) paraformaldehyde (PFA). After washing for 10 min with 50 mM PBS/NH4Cl, ~2x105 cells were seeded in 35 mm IBIDI µ-Dishes (Ibidi GmbH, Martinsried, Germany) coated with 0.05% polylysine. After washing with PBS and blocking for 10 min with 1% BSA in PBS, cells were co-immunostained with anti-glycophorin A (GPA) antibody (1:1000; Abcam, Cambrige UK), as a plasma membrane marker, and with polyclonal anti-PIEZO1 (5µg/mL Abcam, ab82336), then incubated 1h at room temperature. Secondary antibodies for GPA (Alexa Fluor 488 goat anti-mouse A11001; Life Technologies) and for PIEZO1 (Alexa Fluor 546 goat anti-rabbit A11010; Life Technologies) were incubated at 1:200 dilution in PBS for 30 min at room temperature. Cells were mounted in 50% glycerol and 50% PBS and imaged using a Leica TCS SMD FLIM confocal microscope equipped with an oil immersion HCX PL APO CS 63.0 x 1.40 NA, using the following settings: green channel for detecting Alexa488, excitation 488 nm argon laser, spectral range 505-550 nm; red channel for detecting Alexa546, excitation 543 nm DPSS561 laser, spectral range 560-700 nm (by using the meta monochromator). Smears of Ficoll-Hypaque-isolated RBCs isolated were prepared on 35 mm IBIDI µ-Dishes, air dried for 40 min, methanol-fixed for 2 min, then washed four times for 10 min each with PBS. Smears were then co-immunostained with anti-glycophorin A or anti-CD55 (1:100; Abcam, Cambrige UK) and with anti-PIEZO1 antibodies, as described above for CD34+.

Collection and immunohistochemistry analysis of mouse and human embryos

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by the absence of staining in the absence of of primary antibody.

PIEZO1 cloning

Human PIEZO1 cDNA in pF1K Flexi was obtained from the Kazusa human ORF collection. 5'resequencing documented a valid initiation codon. 3'resequencing confirmed the presence of the expected extra C-terminal Val added to the RefSeq sequence by the Kazusa cloning scheme. The Kazusa PIEZO1 cDNA was subcloned into pCRII (Invitrogen) for subsequent mutagenesis. Four-primer PCR mutagenesis was used to restore the natural C-terminal sequence and to introduce individual DHSt-associated missense mutations p.R2488Q and p.R2456H (primer sequences available upon request). The amplified region and ligation boundaries were verified by sequencing after mutagenesis to confirm presence of only the intended mutations and ensure absence of unintended mutations. The WT and mutant PIEZO1 cDNAs were then subcloned into pXT7M for oocyte expression and into pCMV6-Ac-IRES-GFP (OriGene Technologies, Rockville, USA) for mammalian cell expression.

Cell-attached patch clamp of erythrocytes

Blood from patient SF was shipped overnight to Boston on coldpack, buffy coat was removed, cells were passed over cotton wool and washed 5 times in red cell wash solution containing (in mM) 150 choline Cl, 1 MgCl, and 10 Tris-MOPS, pH 7.4, resuspended in storage solution containing (in mM) 145 KCl, 15 NaCl, and 10 HEPES, pH 7.4, then stored at 4°C until use.

Human red cells allowed to settle on coverslips were mounted on an inverted microsope in a 200 µl open chamber (WPI, Sarasota, FL) and superfused 15 min at room temperature by bath solution containing (in mM) 150 Na methanesulfonate, 10 Na EDTA, and 10 Na HEPES, pH 7.4. Borosilicate pipettes (Corning 7052 or 8250) pulled with a Narashigi two-stage puller or a Sutter

P97 puller, then fire-polished to resistances of 10–20 MΩ were front-filled and then backfilled. For study of monovalent cation permeation in the absence of bath Ca^{2+} and Mg^{2+}, symmetric bath and pipette solutions contained (in mM) 150 Na methanesulfonate, 10 Na EDTA, and 10 Na HEPES, pH 7.4.

On-cell (cell-attached) patch currents were recorded with the Axopatch 1-D amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Holding potential was −Vp = −50 mV expressed as the negative of the pipette potential (e.g., equivalent to the intracellular potential with respect to

the pipette). To determine current-voltage relationships (I-V curves) in Fetchex or Clampex (PCLAMP, Molecular Devices, Ca, USA), the realtime control window in gap-free mode was used to record current traces of 10−30 sec duration at holding potentials ranging from −100 to +100 mV, in 25 mV increments. The bath reference electrode was a silver chlorided wire with a 3 M KCl agar bridge. Data was filtered at 500 Hz, digitized at 2 kHz by Clampex, and analyzed offline by Fetchan and Pstat or by Clampfit subroutines of PCLAMP. Holding potentials in on-cell patch experiments were expressed as −Vp, the negative of the pipette potential.

Statistical methods

Student's 2-tailed *t*-test was used to compare differences in quantitative variables. $p<0.05$ was considered statistically significant.

Reference

1. Eisenhoffer GT, Loftus PD, Yoshigi M, Otsuna H, Chien CB, Morcos PA, Rosenblatt J. Crowding induces live cell extrusion to maintain homeostatic cell numbers in epithelia. *Nature*. 2012;484(7395):546-9.

Supplemental Table 1. Identification of the DHSt syndrome gene in family Edinburgh by exome sequencing: number of called variants through sequential filtering steps.

Filtered Variants		
Total variants (SNVs/InDels)	30,435	
Variants called under dominant model *	1,170	
Variations cosegregating with the disease phenotype **	13	
	CDS	**Intron / UTR**
	7	6
Predicted damaging §	5	0

SNVs= Single Nucleotide Variations; InDels= Insertions/Deletions; CDS= Coding Sequence; UTR= Untranslated Region

** Includes the average number per individual of novel single nucleotide non-synonymous variations, splice-site acceptor and donor site mutations, and coding InDels called in heterozygous state.*

*** Includes variations shared by the two affected individuals that are not present in the two unaffecteds from the same family or in a set of 38 control exomes from our internal database.*

§ Predictions generated by automatic queries to Mutation Taster, Poly-phen2 and SIFT.

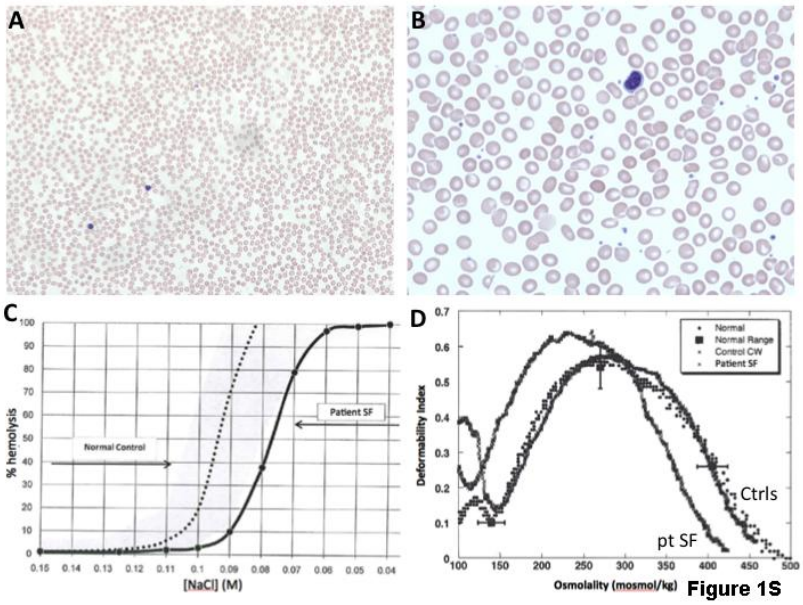


Figure 1S

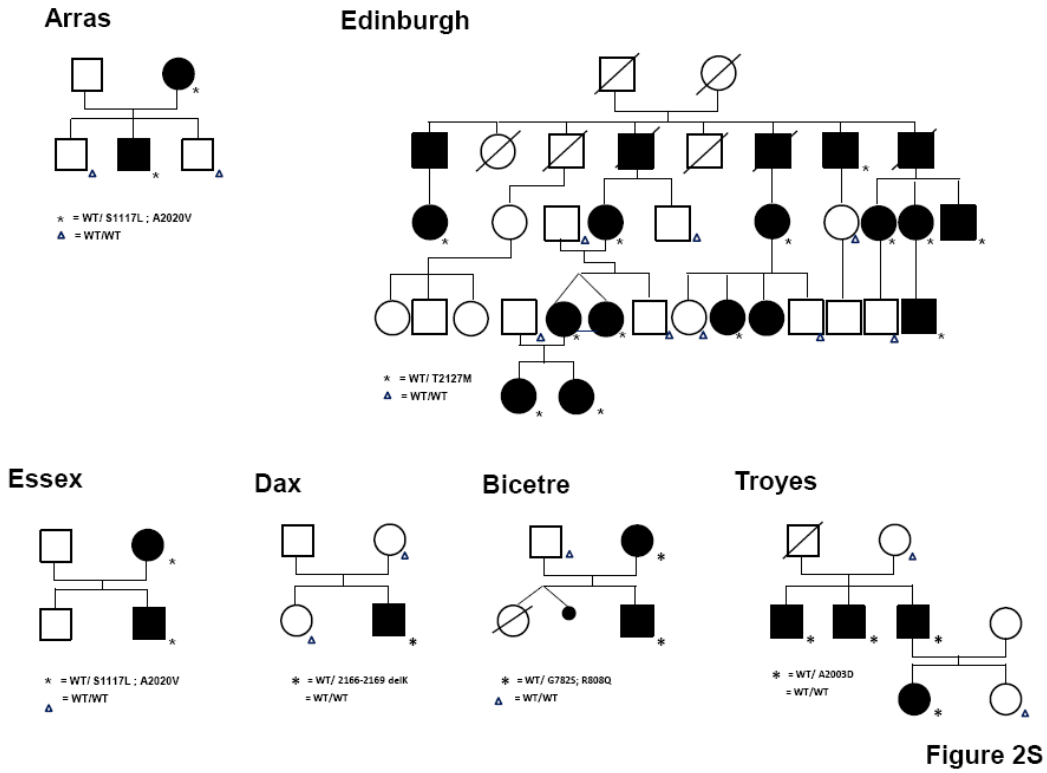


Figure 2S

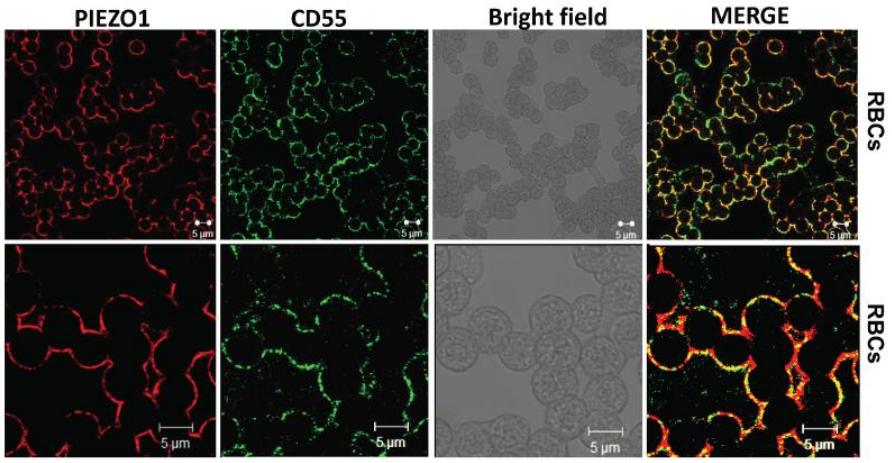


Figure 3S

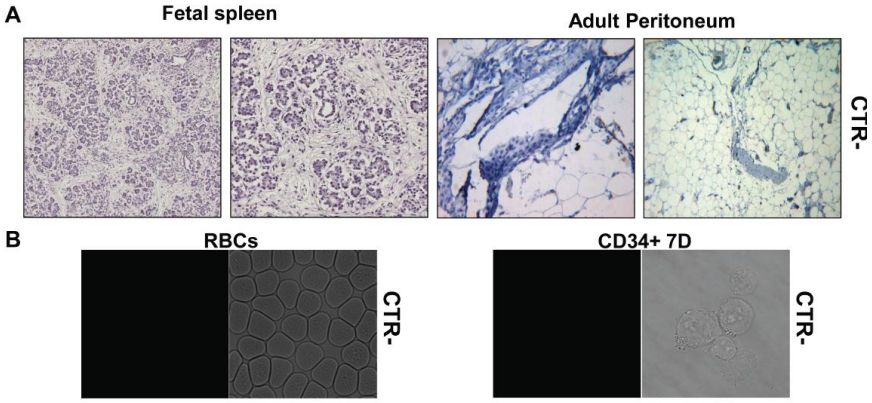


Figure 4S

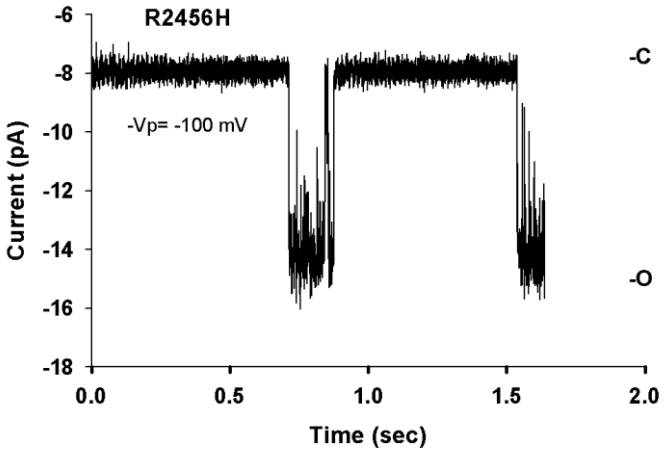
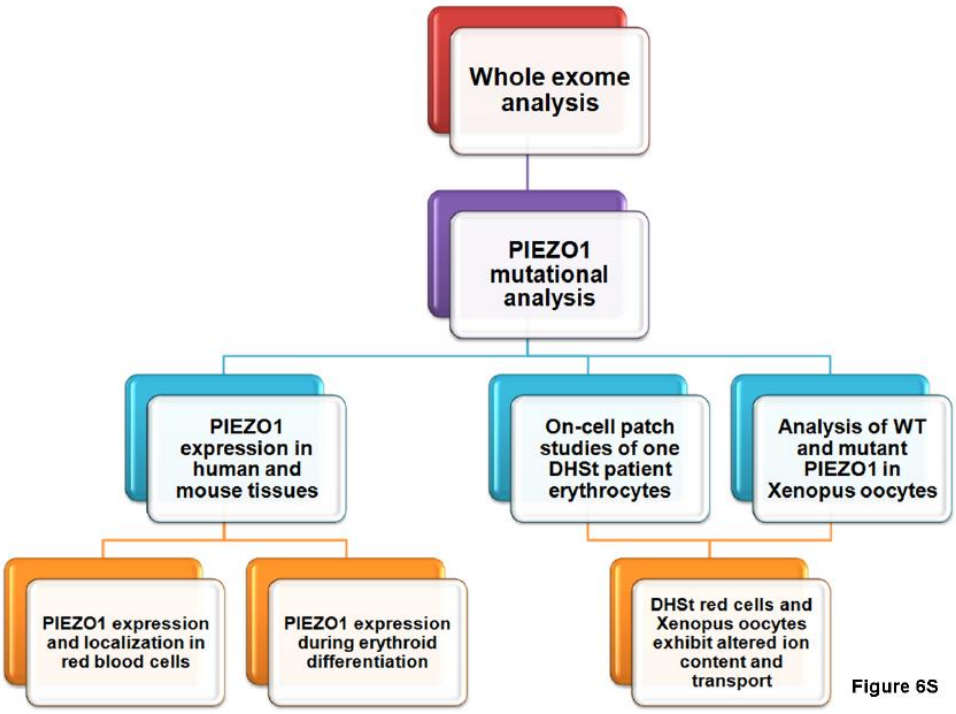


Figure 5S



Supplemental Figure Legends

Figure 1S: Morphology, osmotic fragility, and ektacytometry of red cells from patient SF.

A. Peripheral blood smear of Patient SF (20X). **B.** Higher magnification view of the peripheral blood smear (60x). **C.** Red cell osmotic fragility curve for patient SF and normal control (normal range portrayed in light blue). D. Ektacytometry trace for red cells from pt SF and from a same day control, along with a normal curve for the lab.

Figure 2S: Pedigrees of families analyzed for PIEZO1.

Pedigree of families Arras, Edinburgh, Essex, Dax, Bicetre and Troyes, analyzed for PIEZO1 mutations in this study (modified from literature) [14, 15, 20]. Asterisks indicate patients analyzed for PIEZO1, while those indicated with the triangle are the healthy subjects analyzed as controls in our study.

Figure 3S: PIEZO1 localization in red blood cells

Laser-scanning confocal micrographs of peripheral blood smears showing human red blood cells from a control individual, co-immunostained with rabbit polyclonal antibody to PIEZO1 (red) and

mouse monoclonal antibody to CD55 (membrane marker, green), showing co-localization of the two fluorescence signals (merge). The red blood cells are also shown in bright field. Cells were imaged with a Zeiss LSM 510 Meta confocal microscope equipped with a 1.4 NA oil immersion plan Apochromat 100× objective. Luminosity and contrast were adjusted using the Axiovision software. Representative of three independent experiments.

Figure 4S: Immunohistochemical and immunofluorescence negative controls; and expression of FLAG-tagged PIEZO1 in Hek-293 cells.

A. Negative control of immunohistochemistry on fetal spleen and adult peritoneum (omission of primary antibody). B. Negative control of immunofluorescence on red blood cells (RBCs) and CD34+ at 7 days of erythroid differentiation (omission of primary antibody). C. Immunoblot of PIEZO1 protein in lysate of HEK-293 cells transfected with pCMV6-Ac-IRES-GFP-Flag empty vector and pCMV6-Ac-IRES-GFP-Flag-PIEZO1, as detected with polyclonal anti-PIEZO1 and monoclonal anti-FLAG (50 µg protein; GAPDH was loading control).

Figure 5S: On-cell patch trace from an oocyte expressing PIEZO1 mutant R2456H

A. Cell-attached patch recording from a representative oocyte previously injected with cRNA encoding PIEZO1 mutant polypeptide R2456H, shown at higher temporal resolution than the trace of Figure 6.

Figure 6S: Flow chart showing the study design.

This graph illustrates the design of our study, explaining the combination of genetic investigations and the functional studies.