

Piezo1 plays a role in erythrocyte volume homeostasis

Adèle Faucherre,^{1,2,3} Karima Kissa,⁴ Joël Nargeot,^{1,2,3} Matteo E. Mangoni,^{1,2,3} and Chris Jopling^{1,2,3}

¹CNRS, UMR-5203, Institut de Génomique Fonctionnelle, Département de Physiologie, Labex ICST, Montpellier;

²INSERM, U661, Montpellier; ³Universités de Montpellier 1 & 2, UMR-5203, Montpellier; and ⁴INSERM, UMR-5235, Dynamique des Interactions Membranaires Normales et Pathologiques, Université Montpellier 2, Montpellier, France

ABSTRACT

Mechanosensitivity is an inherent property of virtually all cell types, allowing them to sense and respond to physical environmental stimuli. Stretch-activated ion channels represent a class of mechanosensitive proteins which allow cells to respond rapidly to changes in membrane tension; however their identity has remained elusive. The *piezo* genes have recently been identified as a family of stretch-activated mechanosensitive ion channels. We set out to determine the role of *piezo1* during zebrafish development. Here we report that morpholino-mediated knockdown of *piezo1* impairs erythrocyte survival without affecting hematopoiesis or differentiation. Our results demonstrate that *piezo1* is involved in erythrocyte volume homeostasis, disruption of which results in swelling/lysis of red blood cells and consequent anemia.

Introduction

Mechanosensitivity confers a cell the ability to detect and, ultimately, to respond to a variety of physical stimuli. This attribute relies on the reaction of mechanosensory proteins to mechanical stimuli, such as the stretch-induced opening of an ion channel or force-induced protein conformational changes which disrupt protein-protein interactions.¹ Although electrophysiological recordings of mechanically stimulated cells have indicated the existence of mechanosensitive ion channels, their identity has remained elusive.² The Piezo family of proteins have recently been identified as *bona fide* mechanosensitive ion channels.³ These represent a family of mixed cationic non-selective ion channels gated by mechanical stimuli.^{3,4} Homologs of the *piezo* genes have been identified in a variety of species ranging from protozoa to vertebrates.³ In particular, mammals possess two *piezo* genes, *piezo1* (*fam38A*) and *piezo2* (*fam38B*). Both genes have been detected in a number of tissues in which mechanosensitivity plays a major role, such as kidney, lung and colon, although *piezo1* shows a much higher expression in the skin while *piezo2* expression is elevated in dorsal root ganglion cells.³

Mechanosensitive proteins have been linked to various pathophysiological situations such as hearing and deafness,⁵ cardiac physiological and hypertrophic responses,⁶ as well as kidney function or disease.^{1,7} Current research is now focusing on the functional role of the *piezo* genes in determining the biological processes in which they may be involved.⁸ A recent study in the fruit fly *Drosophila melanogaster* (which has one *piezo* homolog, *Dmpiezo*) has shown that *piezo* has a role in mechanical nociception,⁹ while in-depth genomic analysis has linked the human homolog of *piezo1* to the blood disorder xerocytosis.¹⁰

Given its rapid external development, the zebrafish represents a powerful vertebrate model system for determining gene function during embryogenesis and early development.

Using a morpholino-mediated knockdown approach we sought to elucidate the role of *piezo1* during zebrafish development.

Methods

Zebrafish strains and husbandry

Zebrafish were maintained under standardized conditions and experiments were conducted in accordance with the European Community's council directive of November 1986. Embryos were staged according to Kimmel *et al.*¹¹ The transgenic lines used in this study were the CD41:GFP line, which has been described previously,¹² the MPEG:mCherry line, which was generated in Lutfalla's laboratory based on a previous report,¹³ and the Lmo2:dsRed line, which has been described previously.¹⁴

Morpholinos and injections

Morpholino oligonucleotides were obtained from Gene Tools (Philomath, OR, USA) and injected into one-cell stage embryos. *Pz1 MO1* targets the start codon of *piezo1*, while *Pz1 MO2* targets the splice site at the boundary between intron 1 and exon 2 of *piezo1*. The sequences and amounts of injected morpholino oligonucleotides were as follows:

Pz1 MO1: 5'-ATCCGCATACCACCTGAAGCTCCAT-3'; 8 ng

Pz1 MO2: 5'-AATATGCAGGCTGTGGAAAACACAT-3'; 10 ng

Blood smears

Embryonic blood was obtained by cutting the tail of embryos 2 days post-fertilization (dpf) (10 wild-type embryos and 30 *Pz1* morphant embryos). The blood was collected into a solution of 1x phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 10 U/mL heparin in a microtube and centrifuged for 3 min at 750 rpm. The pellet was resuspended in 5 µL of the same solution, deposited on a slide and air-dried overnight. The blood preparation was then stained with Wright-Giemsa solution following the manufacturer's instructions (Sigma). For immunostaining, blood smears were treated

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Correspondence: chris.jopling@igf.cnrs.fr

as described by Shafizadeh *et al.*¹⁵ Images were taken using a Zeiss AxioImager Z1.

Erythrocyte counting

To count the cells, ten movies of wild-type and ten movies of *piezo1* morphants were taken using a Zeiss stereomicroscope and a 20X M PLAN APO 0.42 objective. For each movie, five time-frames were picked randomly and cells were counted using the Cell-count ImageJ plug-in.

Erythrocyte volume calculation

Wild-type erythrocytes were assumed to be ellipsoid with the diameters of the major and minor axes being equal. The following formula was used to calculate the volume of wild-type erythrocytes.

$$V = \pi/6(abc), a = \text{major axis}, b = \text{minor axis and } c = \text{vertical axis}$$

Piezo1 morphant erythrocytes were assumed to be spherical with equal radii. The following formula was used to calculate the volume of *piezo1* morphant erythrocytes.

$$V = (4/3) \times \pi \times r^3$$

In situ hybridisation

In situ hybridization was performed as described previously.¹⁶

Immunohistochemistry

Embryos were fixed with 4% paraformaldehyde (Sigma) in PBS for 2 h at room temperature, rinsed in PBS-Tween 0.1% and permeabilized by PBS-Tween 0.1%-Triton 1%. Embryos were then blocked for 2 h in 10% sheep serum/PBSDT (PBS, 1% DMSO, 0.1% Triton) at room temperature, incubated overnight with anti- α -tubulin (Sigma, mouse, 1:1000) at 4°C, and washed with PBSDT for 6 h at room temperature. Secondary antibody (Invitrogen, anti-mouse, 1:500) was applied overnight then rinsed in PBS-Tween 0.1% for 4 h and observed by confocal fluorescence microscopy.

Results and Discussion

The zebrafish homologs *piezo1* and *piezo2a* have been previously identified (accession numbers- XP_696355.4 and XP_002666625, respectively³). However, *in silico* analysis using the Ensembl database indicates that a third homolog, *piezo2b* (accession number-XP_003198010), is also present in zebrafish. Gene duplication is not uncommon in zebrafish and is most likely caused by a whole genome duplication event during the animal's evolution.¹⁷ Protein sequence analysis indicates that, overall, zebrafish PIEZO1 shares 40.63% homology with zebrafish PIEZO2A and 46.62% homology with PIEZO2B (Online Supplementary Figure S1). Comparison with its mammalian counterpart indicates that zebrafish PIEZO1 shares 56% homology with human PIEZO1 (Online Supplementary Figure S2). In order to determine the expression of *piezo1* during early development we designed an antisense RNA probe covering the initial 500 bp of this gene. *In situ* hybridization analysis revealed that at 30 h post-fertilization (hpf), *piezo1* is expressed in areas containing primitive erythrocytes, similar to previously reported primitive erythrocyte-specific genes such as *hbbe1*¹⁸ (Figure 1A-E).

To assess the role of *piezo1* during zebrafish development we used antisense morpholino oligonucleotides targeting either the start codon (*piezo1* MO1) or the intron1/exon2 splice site (*piezo1* MO2) of *piezo1*; due to the differences in homology neither of these two morpholinos is predicted to target either *piezo2a* or *piezo2b* (Online Supplementary

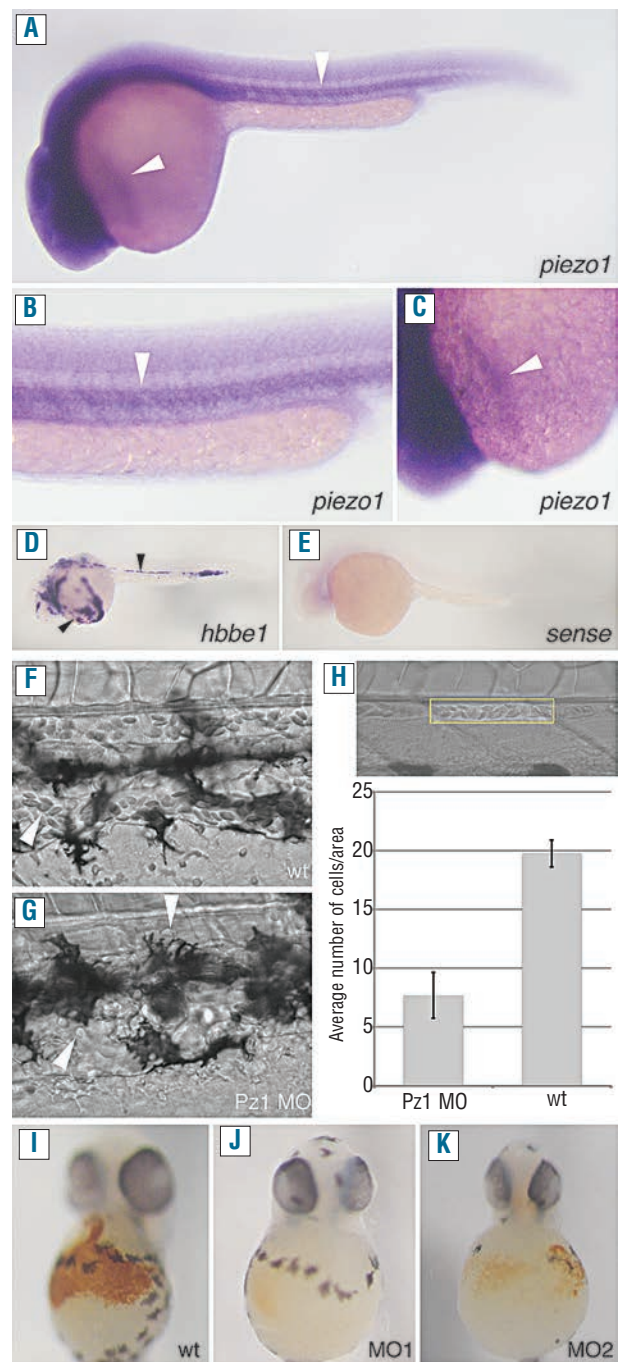


Figure 1. *Piezo1* regulates erythrocyte development/homeostasis. (A-C) *In situ* hybridization analysis using an anti-sense *piezo1* probe. *Piezo1* is expressed in areas containing primitive erythrocytes (indicated by arrows) at 30 hpf. (B,C) Higher magnification of the areas indicated in (A). (D) *In situ* hybridization analysis using an anti-sense *hbbe1* probe labeling differentiated primitive erythrocytes. *Hbbe1* is also expressed in areas containing primitive erythrocytes (indicated by arrows) at 30 hpf. (E) *In situ* hybridization analysis using a sense *piezo1* probe. (F,G) Brightfield images of the dorsal aorta and cardinal vein of 3 dpf wild-type (F) or *piezo1* morphant embryos (G), the arrow heads indicate individual circulating cells. (H) Brightfield image of the area used to count the number of erythrocytes (yellow rectangle). The graph indicates the average number of erythrocytes counted in this region in either wild-type (wt) or *piezo1* morphants (Pz1 MO). (I-K) Two dpf wildtype and *piezo1* morphant embryos treated with the erythrocyte marker o-dianisidine. Wild-type (n=47) (I), *piezo1* MO1 (n=52) (J) and *piezo1* MO2 (n=27) (K). *Piezo1* morphant embryos show a markedly reduced amount of o-dianisidine staining, indicating that erythrocyte development/homeostasis has been disrupted.

Figure S3). We titrated morpholinos to concentrations which did not cause any gross developmental defects/necrosis. Subsequently, we found that *piezo1* morphants displayed a severe reduction in the number of circulating erythrocytes compared to the number of erythrocytes in wild-type controls (Figure 1F-H and *Online Supplementary Movies S1 and S2*). We confirmed this phenotype using *o*-dianisidine, which stains hemoglobin (Figure 1I-K).

To ensure these observations were due to knockdown of *piezo1*, we isolated RNA from *piezo1* MO2 morphants and performed reverse transcriptase polymerase chain reaction analysis. Subsequently we found that the splicing of *piezo1* mRNA transcripts had been disrupted in these embryos, resulting in the observed phenotype (Figure 2A,B). To further verify that the observed phenotype was specific to knockdown of *piezo1* we co-injected suboptimal concentrations of both *piezo1* morpholinos (*Online Supplementary Figure S4*). *O*-dianisidine staining revealed that at sub-optimal concentrations the individual morpholinos had little effect; however, co-injection of both morpholinos at these concentrations produced a marked loss of *o*-dianisidine staining (*Online Supplementary Figure S4*). To understand how knockdown of *piezo1* leads to such a dramatic reduction in the number of circulating erythrocytes, we first investigated whether increased apoptosis could explain this phenotype. However, utilizing the vital dye acridine orange, which stains apoptotic cells,¹⁹ we were unable to

detect any discernible differences between wild-type and *piezo1* morphants, either prior to the onset of circulation (24 hpf) or at later stages (2 dpf) (Figure 2G-N). Another possible explanation for the reduced number of erythrocytes observed in *piezo1* morphants is that primitive hematopoiesis was in some way disrupted. For example, knockdown of the hematopoietic gene *tal1/scf* results in a similar reduction of erythrocytes.²⁰ To investigate this possibility we performed *in situ* hybridization analysis on *piezo1* morphants using a battery of hematopoietic markers, namely: *tal1/scf* (hematopoietic stem cells), *gata1* (early erythrocytes), and *hbbe1* and *band3* (terminally differentiated erythrocytes).¹⁵ We were unable to detect any discernible differences in the expression patterns of these genes when comparing *piezo1* morphants with wild-type embryos (Figure 2C-F). Consequently, it appears that hematopoiesis is unperturbed following *piezo1* knockdown and subsequent erythrocyte differentiation proceeds normally. Taken together these results indicate that the loss of erythrocytes observed in *piezo1* morphants is not caused by either an increase in erythrocyte apoptosis or a perturbation of primitive hematopoiesis, but is most likely due to hemolysis of mature erythrocytes.

We next sought to determine whether *piezo1* is involved in the development/maintenance of other types of blood cells. To do this we injected *piezo1* morpholinos into a number of different transgenic reporter lines (Figure 3A-H). The CD41:GFP transgenic line expresses green fluorescent pro-

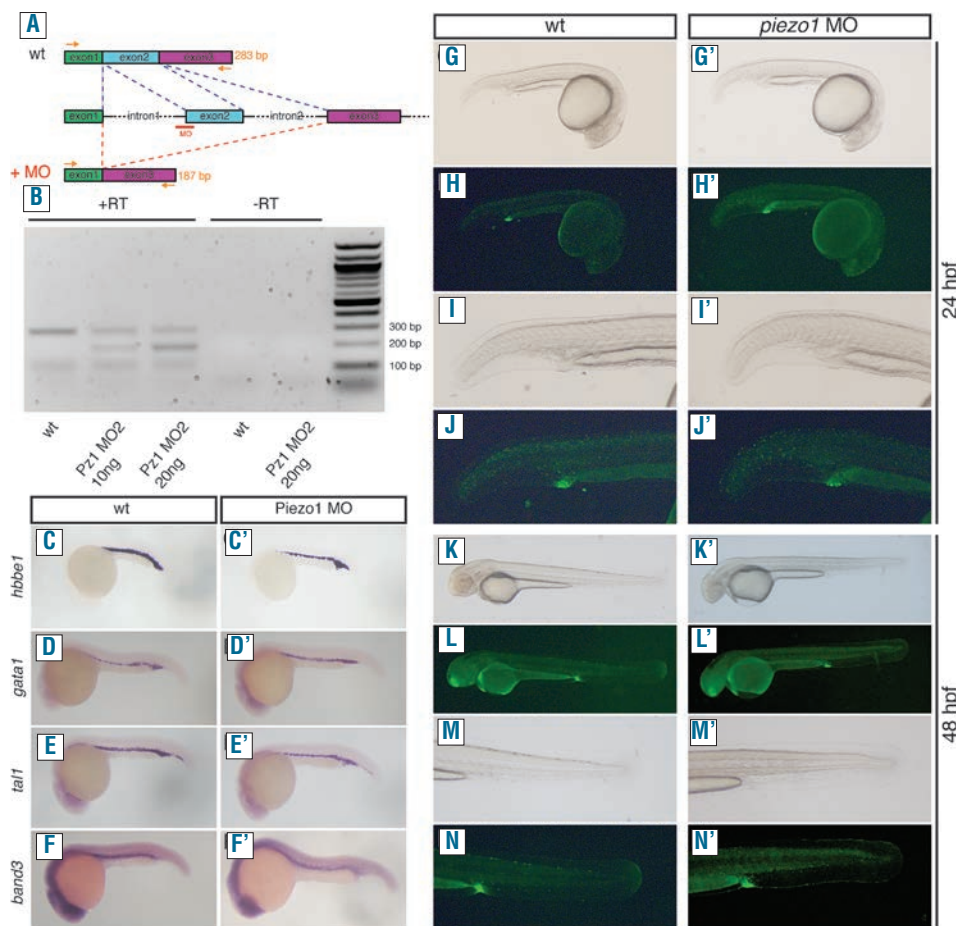


Figure 2. *Piezo1* knockdown does not affect primitive hematopoiesis, erythrocyte differentiation or induce apoptosis. (A,B) RT-PCR analysis of *piezo1* MO2 morphant embryos. The *piezo1* MO2 targets the intron1/exon2 splice site resulting in skipping of this exon (A). Primers designed to the start of exon 1 and the end of exon 3 are able to amplify the correct 283 bp fragment from wild-type embryo cDNA (lane 1). With morphant embryo cDNA the intensity of this 283 bp band is reduced and is accompanied by the appearance of a 187 bp fragment (lane 2), indicating that exon 2 has been excluded. This observation is also dependent on the concentration of morpholino injected (lane 3). Lanes 4 and 5 are negative controls in which no reverse transcriptase was used in the initial reaction indicating that these results are specific to the RNA that has been isolated (B). (C-F') *In situ* hybridization analysis on either wild-type (C-F) or *piezo1* morphant embryos (C'-F'). No differences were observed between wild-type and *piezo1* morphants using probes detecting *hbbe1* (C,C'), *gata1* (D,D'), *tal1/scf* (E,E') and *band3* (F,F'). (G-N) Acridine orange staining of wild-type 24 hpf (G-J) and 48 hpf embryos (K-N). No differences were observed in *piezo1* morphant embryos analyzed at similar time points, 24 hpf (G'-J') and 48 hpf (K'-N'). Images were obtained using an Olympus SZX16 fluorescent microscope.

tein in both thrombocytes (high expression) and hematopoietic stem cells (low expression).²¹ We could not detect any discernible differences in the quantity of either thrombocytes or hematopoietic stem cells in CD41:GFP *piezo1* morphants compared to control embryos (Figure 3C,D), although we did observe a reduction in circulating erythrocytes (Online Supplementary Figure S5). The MPEG:mCherry transgenic line specifically labels the macrophage population in developing zebrafish embryos.¹³ We found that although the concentration of erythrocytes was reduced (Online Supplementary Figure S5), macrophages were still present in MPEG:mCherry *piezo1* morphants, indicating that knockdown of this gene does not affect macrophage homeostasis (Figure 3E,F). To assess whether neutrophils had been affected in *piezo1* morphants we used a previously described Sudan Black stain.²² We did not detect any differences in the neutrophil population between wild-type and *piezo1* morphant embryos (Figure 3G,H). Taken together these data suggest that loss of *piezo1* does not have any significant effect on the development/homeostasis of other blood cell lineages. In order to ascertain what was causing the loss of erythrocytes observed in *piezo1* morphants, we injected *piezo1* morpholinos into a Lmo2:dsRed reporter line which specifically labels erythrocytes and blood vessels.¹⁴ In control embryos we detected circulating erythrocytes with a typical flattened elliptical morphology²³ (Figure 3I,J). Conversely, the few remaining erythrocytes we were able to detect in Lmo2:dsRed *piezo1*

morphants had a spherical morphology with obvious signs of membrane deformation (Figure 3K,L). Further analysis showed that the dimensions of *piezo1* morphant erythrocytes had shifted from an elliptical 3:1 ratio (average diameter=9.29 $\mu\text{m} \pm 1.16$ SD, average height=2.89 $\mu\text{m} \pm 0.62$ SD, n=50) to a spherical 1:1 ratio (average diameter=7.48 $\mu\text{m} \pm 0.5$ SD, average height=7.45 $\mu\text{m} \pm 0.58$ SD, n=50) indicating that knockdown of *piezo1* results in an increase in erythrocyte volume (average wild-type erythrocyte volume=130 μm^3 , average *piezo1* morphant erythrocyte volume=219 μm^3) (Figure 3I,K). To investigate these observations further, we analyzed blood smears prepared from wild-type or *piezo1* morphant embryos. Wright and Giesma stained blood from *piezo1* morphant erythrocytes appeared markedly different from that of their wild-type counterparts, having deformed/ruptured plasma membranes and swollen nuclei (Figure 4A-D.). Furthermore, immunohistochemical analysis using an α -tubulin antibody to label the marginal band of microtubules, phalloidin (actin cytoskeleton) and Hoechst (nucleus) confirmed these observations (Figure 4E-L). The cellular morphology observed in these *piezo1* morphant erythrocyte preparations differed somewhat from the spherical/deformed shape we had observed *in vivo*. We surmised that this may be due to an increase in fragility of these cells, such that the preparation of the blood smear may have induced the observed rupture/lysis. To circumvent this possibility we performed similar immunohistochemistry analyses on whole mount *piezo1* morphant

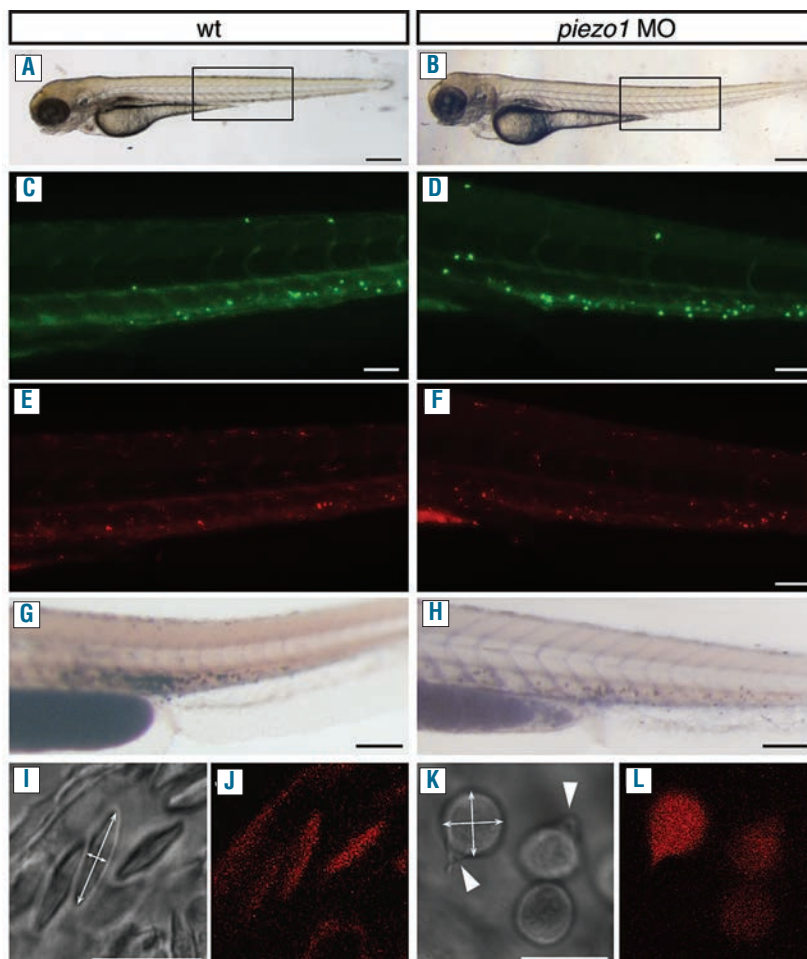


Figure 3. *Piezo1* knockdown perturbs erythrocyte volume homeostasis without affecting other blood cell lineages. (A,B) Brightfield images of either wild-type or *piezo1* morphants, the black box indicates the areas depicted in (C-H). CD41:GFP expression in either wild-type (n=20) (C) or *piezo1* morphants (n=32) (D). Thrombocytes have high GFP expression and hematopoietic stem cells have low GFP expression. MPEG:mCherry expression in either wild-type (n=20) (E) or *piezo1* morphants (n=32) (F). Macrophages are labeled with mCherry. Sudan Black staining of either wild-type (n=15) (G) or *piezo1* morphants (n=17) (H). Sudan Black labels the neutrophil population. Brightfield and fluorescent images of erythrocytes in either wild-type Lmo2:dsRed (I, J) or *piezo1* morphant Lmo2:dsRed embryos (K, L). Double-headed arrows in (I, K) indicate how erythrocyte dimensions were calculated: the arrow heads in (K) indicate membrane deformation. Scale bars: A-B 150 μm , C-H 35 μm and I-L 10 μm .

embryos obtained from the *Lmo2:dsRed* transgenic line. This indicated that although *piezo1* morphant erythrocytes appear spherical with signs of membrane deformation, they do not have such a dramatic ruptured phenotype as that observed in the blood smear preparation (Figure 4M-R). The marginal band of microtubules appears to be disrupted when compared to that of the control erythrocytes. Rather than encircling the erythrocytes in a single plane it appears more diffuse (Figure 4M,P). Taken together these results indicate that knockdown of *piezo1* leads to erythrocyte swelling. These cells also adopt a spherical shape and exhibit signs of membrane deformation, along with disruption of the marginal band of microtubules.

Here we have shown that knockdown of *piezo1* leads to a drastic reduction in the number of circulating erythrocytes, which is not due to perturbed primitive hematopoiesis or erythrocyte differentiation. Our observations indicate that *piezo1* morphant erythrocytes become spherical and display a disrupted marginal band of microtubules, similar to observations made in zebrafish models of spherocytosis.^{15,24} When components of the erythrocyte cell membrane, such as protein 4.1 or β spectrin, are disrupted, this leads to a reduction in the cell surface (due to membrane blebbing) and failure to maintain an elliptical shape. Consequently, this results in the contraction of erythrocytes to the spherical form observed in spherocytosis.²⁵ However, although *piezo1* morphant erythrocytes adopt a spherical shape they appear to be swollen, increasing their overall volume in the process. Furthermore, we did not observe the increased apoptosis associated with spherocytosis, which indicates that the phenotype observed in *piezo1* morphants is more akin to conditions such as hydrocytosis.^{24,26} Osmotic induced swelling of erythrocytes leads to changes in cell membrane tension.²⁷ This will induce the stretch-activated channel *piezo1* to open and ultimately counteract this increase in water influx to avoid erythrocyte lysis. If, however, *piezo1* is missing, the erythrocyte will continue to swell and ultimately lyse. Interestingly, we observed that the marginal band of microtubules was disrupted in *piezo1* morphants. Although we cannot rule out that this is just a consequence of swelling-induced lysis, it may indicate that *piezo1* also has a structural role, similar to the anion exchanger *band3*. Indeed, observations similar to those in *piezo1* morphants have been made in frog red blood cells. Frog erythrocytes can be induced to swell using specific toxins that induce water influx. However, these cells still retain an elliptical form. Subsequent disruption of the cytoskeleton using cytochalasin D leads to the development of a swollen spherical shape prior to lysis.²⁸ Thus the defects associated with knockdown of *piezo1* may also be due, in part, to disruption of the cytoskeleton.

Recent reports have linked *piezo1* to the hereditary blood disorder xerocytosis in humans, and established that *piezo1* is present in the membranes of erythrocytes.^{10,29} Xerocytosis is an autosomal dominant hemolytic anemia characterized by a defect in the cell membrane leading to potassium efflux and resulting in rigidity, hemolysis and reduced erythrocyte survival.³⁰ However, our data suggests that loss of *piezo1* results in erythrocyte swelling and lysis which is more consistent with conditions such as hydrocytosis rather than the dehydration observed in xerocytosis.^{31,32} Interestingly, it has recently been established that the mutations in *piezo1* associated with xerocytosis lead to delayed channel inactivation. This means the channel will stay open for a longer time, resulting in dehydrated erythrocytes

rather than the swollen cells we observed when *piezo1* is knocked down.^{33,34}

Undoubtedly, the severe phenotype we observe in *piezo1* morphants would be lethal in mammals and it is unlikely that complete disruption of this gene could be tolerated. However, mutations may exist which could reduce the

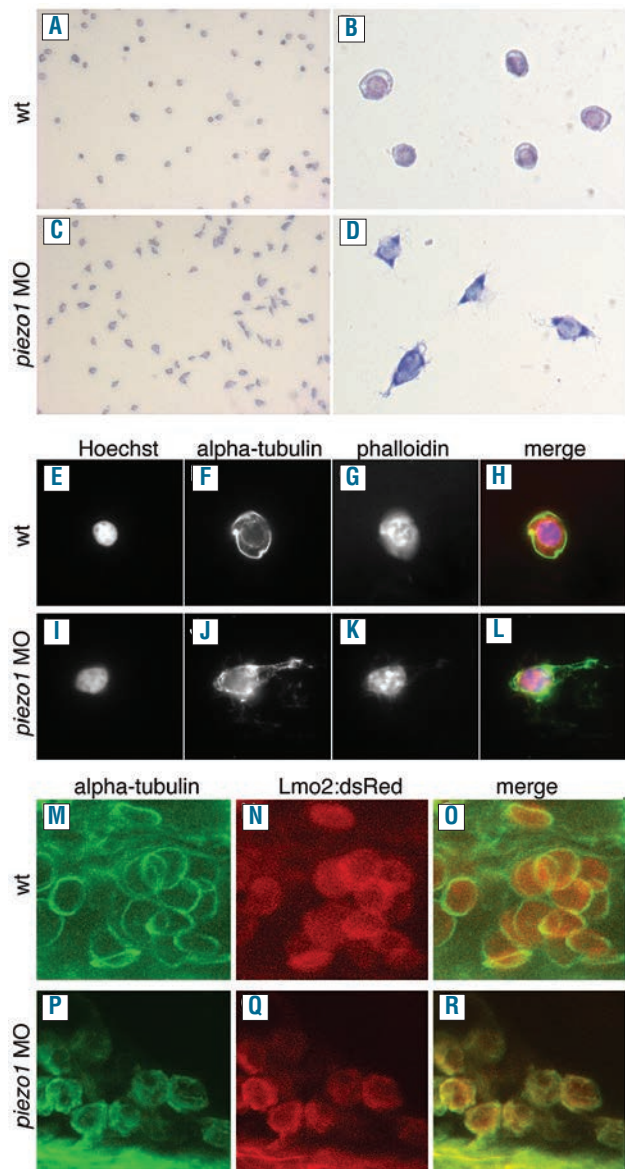


Figure 4. *Piezo1* knockdown results in deformed erythrocytes and disruption of the marginal band of microtubules. (A-D) Wright-Giesma stained blood smears prepared from wild-type (A,B) and *piezo1* morphant embryos (C,D). *Piezo1* morphant erythrocytes appear deformed with signs of membrane rupture and swollen nuclei (D). Immunohistochemistry analysis of either wild-type (E-H) or *piezo1* morphant embryos (I-L), utilizing Hoechst to stain the nuclei (E,I), an anti- α -tubulin to label the marginal band of microtubules (F,J) and phalloidin to label the cytoskeleton (G,K) and merged images (H,L). *Piezo1* morphant erythrocytes have swollen deformed nuclei (I) and a deformed/ruptured membrane (J). *In vivo* immunohistochemistry analysis of either wild-type (M-O) or *piezo1* morphant embryos (P-R) obtained *Lmo:dsRed* transgenic zebrafish. The marginal band is labeled with anti α -tubulin (M,P) and erythrocytes express *dsRed* (N,Q). *Piezo1* morphant erythrocytes appear spherical and have a disrupted/diffuse marginal band of microtubules (P,R) when compared to control erythrocytes in which the marginal band encircles the erythrocytes in a single plane (M,O).

functionality of the channel, resulting in the swelling of erythrocytes associated with conditions such as hydrocytosis. Furthermore, if *piezo1* also plays a structural role, there may also be mutations that affect this function while still retaining an operational channel, resulting in a phenotype more akin to spherocytosis.

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Authorship and Disclosures

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