The mechano-sensitive ion channel Piezo mediates Rho activation and actin stress fibre formation in *Drosophila* nephrocytes

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Abstract

Mechanotransduction is an important process of sensing physical forces in the environment of organisms, tissues and cells and transducing them into a biochemical response. Due to their position on the glomerular capillaries, podocytes are exposed to near-constant biomechanical force, which can fluctuate widely. These include shear stress and hydrostatic pressure. A pathological increase in these forces can induce morphological change to podocytes, their detachment from the glomerular basement membrane and subsequent loss into the primary urine. The ability to sense and respond to variations in mechanical force would be beneficial to a cell exposed to these conditions. It is likely podocytes have such mechanisms, however their identity are unknown.

Here we investigated the hypothesis that the mechanotransducer Piezo is involved in a mechanotransduction pathway in *Drosophila* nephrocytes, the podocyte homologue in the fly. We find Piezo is expressed in nephrocytes and localizes to the nephrocyte diaphragm. The Piezo agonist YODA, which stimulates channel opening in the absence of mechanical force, leads to a significant increase in intracellular Ca^{++} upon

shear stress in the nephrocyte. This leads to activation of Rho1, delineating a putative Piezo mechanotransductive pathway in these cells.

Loss of function analysis revealed minor defects in nephrocyte filtration function. In contrast, we show that elevated Piezo levels resulted in constantly oscillating Ca^{++} signals even in the absence of shear stress, increased active Rho1 and accumulation of actin stress fibers, culminating in a severe nephrocyte filtration phenotype, suggesting that pathway hyperactivity is detrimental. We asked if this phenotype could be reversed by blocking Piezo activity pharmacologically using the tarantula toxin GsMTx4. Treatment with GsMTx4 brought levels of activated Rho1 into the normal range.

This work delineates a mechanotransductive pathway in nephrocytes involving Piezo, Ca^{++}, Rho1 and the actin-cytoskeleton, and suggest this is part of a mechanism by which nephrocytes sense and adapt to changes in mechanical force.

Introduction

Kidney podocytes are highly specialized epithelial cells, which together with the fenestrated endothelium and the glomerular basement membrane, constitute the three-layered, size and charge selective filter of the glomerular filtration barrier [1]. Podocytes have a unique morphology, forming primary and secondary foot processes which completely enwrap the capillaries. Injury of podocytes causes severe morphological change, detachment from the basement membrane and finally loss of podocytes into primary urine. Until today several genes are described to be important for podocyte biology, among them are NPHS1, NPHS2, TRPC6 and Actinin-4, to name only a few [2–6]. Loss of podocytes has pathological consequences as these cells are post-mitotic and cannot be replenished. The blank capillaries are not sealed anymore

resulting in massive loss of protein from blood into urine, so called proteinuria. Podocyte injury can occur because of genetic deficiencies [2–6], toxin-mediated triggers [7] and changes in the physical environment within the glomerulus [8–11].

Podocytes are under constant mechanical force and regularly face changes in in these forces, as they are responsible for the filtration of ~180 litres of primary urine each day. The main forces arising from this are hydrostatic pressure (50 kPa) and fluid shear stress at the filtration slits (8 Pa) [12]. These forces increase during diseases such as diabetes and hypertension and result in podocyte loss [10]. Progressive loss of podocytes leads to a vicious cycle as biomechanical forces experienced by those cells remaining increases further. It is known remaining podocytes respond by growing in size to re-cover the blank capillary to overcome the challenge originating from loss of neighbouring cells; this is considered to be an adaptive and protective mechanism [13–15]. In order for this to happen, podocytes must exhibit mechnotransductive mechanisms to recognize these changes in the physical environment and initiate adaptive and protective responses, likely candidates are TRPC6, YAP, Talin and Filamins and Piezo [5,8,9,16–19].

Piezo is a mechanosensitive ion channel that is involved in mechanotransduction in many development and physiological contexts. Piezo channels (Piezo1 and Piezo2) open in response to mechanical force and allow the influx of positively charged ions such as Ca^{++} and Na^{+} [20,21]. The two proteins have different functional roles as Piezo1 was reported to be essential for sensing blood flow-associated shear stress and blood vessel development [22–24], while Piezo2 responds to touch and proprioception [25]. Piezo is expressed in podocytes however it is unknown if it is involved in a mechanotransduction pathway in these cells, and if so how it might function [19].

To address these questions, we used the *Drosophila* nephrocyte model and investigated the effect of Piezo depletion and activation on nephrocyte morphology and function. Nephrocytes form foot processes, which are spanned by the nephrocyte diaphragm, a highly-specialized cell-cell contact, that is highly similar to the mammalian filtration barrier in the glomeruli [26,27]. They function as filtration units in the fly and filter toxins and waste products from the haemolymph. Our data show that nephrocytes respond to shear stress with an increase of intracellular Ca^{++} levels. Moreover, we could show that the mechanotransducer Piezo is expressed in nephrocytes and localizes to the nephrocyte diaphragm. Piezo loss of function results in a mild phenotype. In contrast hyperactivation of Piezo caused severe filtration defects, significant activation of Rho1 and accumulation of actin stress fibers. Thus, our data confirms, that Piezo is involved in a mechanotransduction pathway in nephrocytes and its hyperactivity is detrimental for nephrocyte function.

Material and Methods

Table 1: List of antibodies

Name	**Company/ Provider**	**Catalog nr. / Reference**	**Host Species**	**Dilution IF**
Duf	M. Ruiz-Gomez	[26]	Rabbit	1:100
Pyd	Developmental Studies Hybridoma Bank	PYD2	Mouse	1:25

Table 2: List of fly strains

Fly strain	**Origin**	**Purpose**	**Chromosome**

Piezo MI{MIC}	BDSC ID: 60209	Endogenous GFP-tag	2. Chr.
Piezo^{ko}	BDSC ID: 58770	Knockout	2. Chr.
UAS-*piezo*-GFP	BDSC ID: 58773	Overexpression	3. Chr.
UAS-*piezo*-RNAi	VDRC ID: 101915	Knockdown	2. Chr.
UAS-*sns*-RNAi	VDRC ID: 109442	Knockdown	2. Chr.
UAS-*duf*-RNAi	VDRC ID: 27227	Knockdown	2. Chr.
sns-Gal4; UAS-*dicer*2	[8]	Nephrocyte-specific driver	2. + 3. Chr.
UAS-Rok-RBD-GFP	BDSC ID: 52290	Rho1 sensor	3. Chr.
UAS-Pak-RBD-GFP	BDSC ID: 56550	Rac/Cdc42 sensor	3. Chr.
UAS-LifeAct-RFP	BDSC ID: 58362	Overexpression	3. Chr.
UAS-GCamP6medium	BDSC ID: 42750	Overexpression	3. Chr.
Oregon R		wildtype	
W^{1118}		wildtype	

Fly husbandry and generation

For experiments all flies were kept at 25°C. The nephrocyte-specific knockdown or overexpression was achieved by mating UAS fly strains with the *sns*-Gal4 strain.

YODA and GsMTx4 treatment

To active Piezo acutely nephrocyte were incubated with the specific activator compound YODA (10nm in DMSO; control: DMSO) (Sigma, now Merck). To inhibit mechano-sensitive channels in an unspecific way nephrocytes were incubated with tarantula toxin/GsMTx4 (5mM in H_2O; control: HL3 buffer) (Alomone labs, Jerusalem, Israel, Cat. nr: STG-100). For all experiments nephrocytes were isolated as described below in haemolymph-like buffer (HL3.1; 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM $NaHCO_3$, 115 mM Sucrose and 5 mM Hepes). Cells were then

incubated for 5 mins followed by a washing step in HL3 for one min. Nephrocytes were fixed for 20 mins with 4 % formaldehyde before mounting.

Immunofluorescences of *Drosophila* tissue

Garland nephrocytes were isolated from 3rd instar larvae in HL3 buffer. After isolation cells were fixed in 4 % formaldehyde for 20 mins, followed by three washing steps with washing buffer (Phosphate buffer saline, 0.5% bovine serum albumin, 0.03% Triton-X) for 20 mins. Primary antibody incubation was done overnight at 4°C. On day 2, the cells were washed three times for 20 mins, incubated with 5 % normal horse serum for 30 min and 1 h with the respective secondary antibody. Afterwards followed three washing steps for 20 mins and mounting with mounting medium (2.5% propyl gallate and 85% glycerol). Imaging was done using either a Zeiss LSM 8 confocal combined with an Airyscan for higher resolution or a Leica SP8 confocal. Images were further processed using ImageJ (version 1.53c).

FITC-Albumin uptake assay

FITC-Albumin uptake assays were performed as previously published [8,28]. Garland nephrocytes were isolated from 3rd instar larvae and incubated with FITC-Albumin (0,2 mg/ml; Sigma-Aldrich, St. Louis, USA) for 1 min, followed by 20 mins fixation with 4 % Formaldehyde. Imaging was done with a Zeiss LSM 5 confocal or Leica SP8 confocal microscope and images were further processed and analyzed using ImageJ (version 1.53c). For comparative analysis, the exposure time of experiments was kept identical. The mean intensity of FITC-Albumin was measured and normalized to control cells. Images were also used to quantify nephrocyte size by measuring the area of each cell.

$AgNO_3$ toxin assay

Flies of the appropriate genotype were allowed to lay eggs for 24 hrs at 25°C on juice plates with normal yeast. Afterwards, plates with eggs were kept at 18°C for 24 hrs. 1st instar larvae were then transferred to juice plates supplemented with yeast paste containing $AgNO_3$ (2 g yeast in 3.5 ml 0.003% $AgNO_3$). Plates were kept at 25°C for 4 days to let larvae develop and pupate. 5 days after egg laying pupae were counted daily for 4 consecutive days. The number of pupae was normalized to the number of 1st instar larvae transferred to the plates.

Quantification of GTPase activity levels

To quantify activity levels of Rho1 and Rac/Cdc42 in garland nephrocytes. UAS-Rok-RBD-GFP and UAS-Pak-RBD-GFP flies were combined with the nephrocyte-specific driver *sns*-Gal4. For final matings, these flies were either combined with UAS-*piezo*-RNAi or UAS-*piezo*-GFP flies. Garland nephrocytes from 3rd instar larvae were isolated and fixed for 20 mins with 4 % Formaldehyde followed by mounting with mounting medium. Images were obtained using a Leica SP8 confocal and further image processing was done using ImageJ (version 1.53c). The mean GFP intensity was measured and normalized to cell size prior to normalization to control cells. For comparative analysis, laser intensity and settings were kept equal.

Visualization of the actin filaments

For actin cytoskeleton visualization, the nephrocyte-specific driver *sns*-Gal4, UAS-LifeAct-RFP and UAS-Piezo-GFP flies were combined. Garland nephrocytes were isolated from 3rd instar larvae and fixed for 20 mins with 4 % Formaldehyde, prior to mounting with mounting medium. Images were obtained using a Leica SP8 confocal. Further image processing was done using ImageJ (version 1.53c). For quantification

mean intensities of RFP were measured and normalized to cell size. For comparative analysis, laser intensity and settings were kept equal.

Ca^{++} imaging of pericardial nephrocytes

For Ca^{++} imaging purposes UAS-GCamP6 flies were combined with the nephrocyte-driver *sns*-Gal4 and mated with UAS-*piezo*-RNAi or UAS-*piezo*-GFP to obtain experimental animals. Flies were kept at 25°C until the experimental flies were 1-5 days old. For *ex vivo* Ca^{++}-imaging only male flies were used. Pericardial nephrocytes were isolated, but still attached to the heart tube and the cuticle. The tissue was kept in HL3.1 buffer. For imaging a Zeiss Axio Examiner microscope with a x40 dipping objective, fitted with GFP filters and LED light source (Carl Zeiss Ltd.) and connected to a Prime 95B sCMOS camera (Photometrics) running from Micro-Manager 1.4 software was used. Images were acquired every second for 150 secs and after 60 secs a wave was induced by pipetting HL3.1 buffer into the dish with the tissue. For quantification of the GCamP6 signal a previously published FIJI macro was used [29]. For comparative analysis, the mean of the first 10 measurements of every cell was used for quantification ($F/_{F1-10}$). To quantify the signal amplitude parameters (height and width), values after normalization were used. To determine baseline values raw data was used.

Statistical analysis

To determine statistical significance Graph Pad Prism software version 8 for Mac (GraphPad Software, San Diego, CA) was used. All results are expressed as means ± SEM. Comparison of two groups was done using a student's t-test. A *P-value* < 0.05 was considered statistically significant. Comparison of more than two groups with one

independent variable was done using one-way ANOVA followed by Tukey's multiple comparisons test. A *P-value* < 0.05 was considered statistically significant.

Results

Drosophila nephrocytes respond to shear stress by Ca^{++} influx

Podocytes rely on Ca^{++} as a second messenger, but increased and prolonged Ca^{++} influx results in podocyte injury and loss, as seen for gain-of-function mutations of the cation channel TRPC6 [5,30]. Here, we wanted to test the hypothesis that nephrocytes also rely on Ca^{++} as second messenger and whether they respond to shear stress by opening of mechano-sensitive channels, which subsequently results in Ca^{++} influx.

We isolated pericardial nephrocytes from adult flies, which express the Ca^{++} sensor GCAmP6m. The cells were incubated in haemolymph-like buffer for imaging. We induced shear stress by washing a stream of buffer over the cells. This induction of shear stress resulted in an immediate increase of the Ca^{++} signal intensity (GCAmP6m) in nephrocytes **(Figure 1A third image and B left panel)**. This data confirms that nephrocytes respond to shear stress with an immediate single Ca^{++} transient. This is consistent with the opening of surface mechanosenstive ion channels resulting in Ca^{++} influx.

We next asked, if this mechanism is disturbed in injured nephrocytes. To induce injury, we depleted nephrocytes for Duf (dNEPH) or Sns (dNephrin), which results in a severe morphological and functional phenotype in nephrocytes [26,27]. The Ca^{++} transient amplitude (signal intensity) and signal duration after shear stress was significantly reduced in Duf- or Sns-depleted nephrocytes **(Figure 1A,B,C,D)**. In addition, GCamP6 baseline intensity was significantly reduced **(Figure 1E)**,

suggesting a major perturbation of Ca^{++} signalling upon nephrocyte injury. Our data also confirms that nephrocytes respond to shear stress by Ca^{++} influx, which is consistent with the hypothesis that mechanotransduction is an inherent property of nephrocytes.

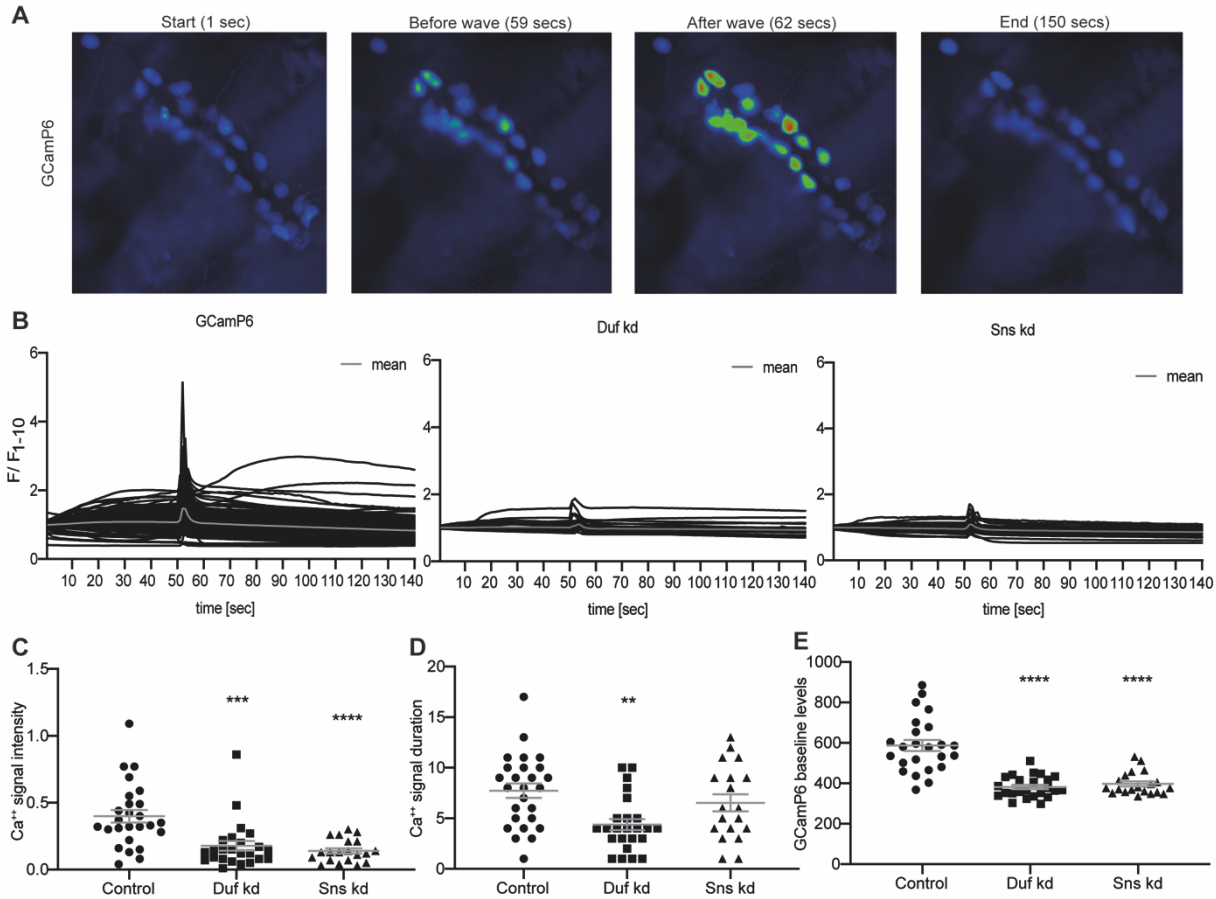

Figure 1: Loss of the nephrocyte diaphragm proteins Sns and Duf results in disturbances of Ca^{++} signalling. A,B Ca^{++} signalling events were recorded for 150 secs. After 60 secs shear stress was induced by adding buffer and an immediate Ca^{++} signal was recorded, which is depicted by the peak in the graph. This peak was significantly smaller in Duf and Sns knockdown nephrocytes, when compared to GCAmP6 controls. Control: w; *sns*-Gal4/+;UAS-*GCAmP6m*/+; Duf kd: w; *sns*-Gal4/ UAS-*duf*-RNAi;UAS-*GCAmP6m*/+; Sns kd: w; *sns*-Gal4/ UAS-*sns*-RNAi;UAS-*GCAmP6m*/+. **C** Peak Ca^{++} signal intensity was significantly reduced in the knockdown cells. 1way ANOVA with Tukey's multiple comparison test: ***: $p < 0.001$; ****: $p < 0.0001$. **D** Peak duration was also significantly reduced after depleting Sns and Duf. 1way ANOVA with Tukey's multiple comparison test: **: $p < 0.01$. **E** Baseline GCAmP6 intensity was also measured and showed a significantly reduced intensity in the knockdown cells, when compared to controls. 1way ANOVA with Tukey's multiple comparison test: ****: $p < 0.0001$.

Piezo is expressed in nephrocytes and localizes to the nephrocyte diaphragm

The mechanotransducer Piezo opens upon mechanical force, leading to the influx of Ca^{++} (and other cations) and is therefore a candidate for this mechanism. It

was reported that Piezo is expressed in podocytes, but the exact localisation pattern remains unclear for now [19]. Here, we first examined Piezo expression in nephrocytes and could show co-localisation with the nephrocyte diaphragm proteins Duf, the NEPH homolog in the fly, and Pyd (dZO1), suggesting Piezo is a component of the nephrocyte diaphragm multi protein complex **(Figure 2A)**. A previous study revealed Piezo1 expression in mouse glomeruli [19], further supporting our hypothesis that Piezo is expressed in podocytes and nephrocytes and is involved in mechanotransduction pathways not only in *Drosophila*, but also mammals. High resolution microscopy of Duf and Pyd at the surface of the nephrocyte revealed a fingerprint-like pattern highlighting the nephrocyte junction. At this resolution, Piezo showed strong co-localisation with Duf; Pyd does not fully overlap with Piezo but strongly echos its pattern. These data reinforce the hypothesis that Piezo is a component of the nephrocyte diaphragm. It remains to be tested whether there is direct interaction between any of these proteins.

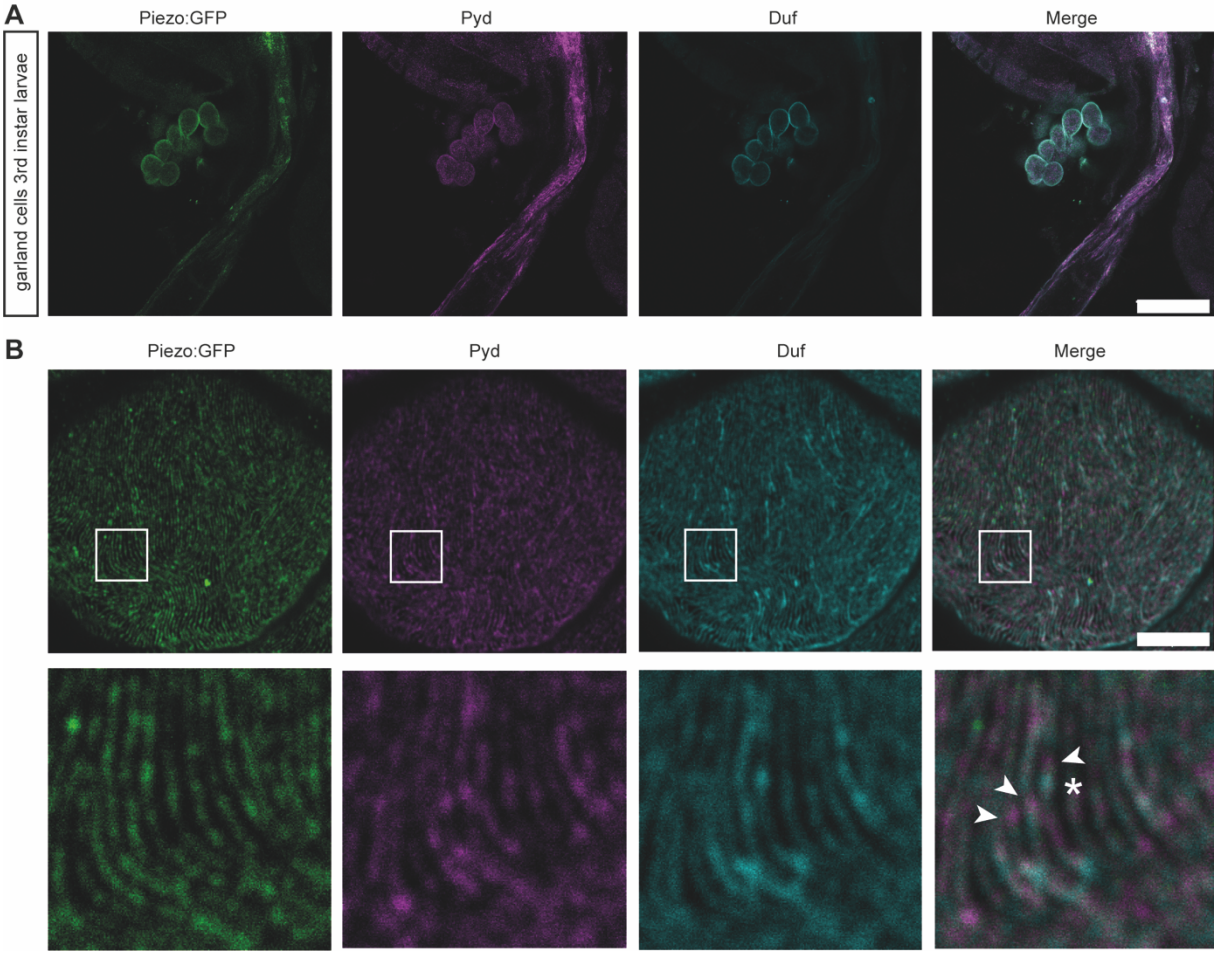

Figure 2: Piezo is expressed in nephrocytes and localizes to the nephrocyte diaphragm. A Immunofluorescence staining with a Duf (dNEPH) and Pyd (dZO1) antibody revealed expression and localisation of Piezo (Piezo:GFP; Mi{MIC} line) at the nephrocyte diaphragm in garland nephrocytes from 3rd instar larvae . Scale bar = 50 μm. **B** High resolution microscopy showed co-localisation of Piezo and Duf, while Pyd presented with an adjacent localisation. Arrow heads: Pyd, Star: Piezo and Duf. Scale bar = 5 μm.

Pharmacological activation of Piezo with YODA results in increased Rho1-GTP levels

Further, we investigated whether pharamcological activation of Piezo has an impact on nephrocyte biology, confirming a functional role of the channel in these cells. To do so, we used YODA, which is a chemical compound which specifically facilitates Piezo opening at lower magnitude of force [31,32]. As Piezo is a Ca^{++} permeable cation channel and known to be important for Ca^{++} influx, we investigated the effect of YODA by measuring Ca^{++} levels in nephrocytes. Piezo channels are sensitive to mechanical

stimuli and stretch, hence here we induce shear stress and measured the immediate Ca^{++} signal intensity (GCAmP6 peak), duration and the baseline GCamP6 levels.

5-minute incubation of pericardial cells with YODA resulted in a significant increase of both, the amplitude (signal intensity) and signal duration, after induction of shear stress and when compared to DMSO incubated cells (controls) **(Figure 3A)**. Interestingly, baseline levels of GCamP6 intensity (before applying shear stress) were significantly lower in cells incubated with YODA, suggesting that potential compensatory pathways of Ca^{++} efflux might be activated. The increased Ca^{++} signal intensity and duration upon incubation with YODA (a Piezo-specific agonist) provides additional support for a Piezo-dependent mechanotransduction mechanism in nephrocytes. Moreover, it also supports the hypothesis that Piezo is involved in a mechanotransduction pathway in nephrocytes which is mediated via Ca^{++} influx. We investigated whether the YODA mediated Piezo sensitization had short-term phenotypic consequences on nephrocyte morphology and filtration function. Analysis after a 5 minute incubation with YODA did not cause any changes in morphology of the filter slit, nor filtration function as assessed with the FITC Albumin uptake assay, when compared to DMSO incubated controls **(Figure 3B,C)**.

Ca^{++} is a second messenger in cells and can act by modulating GTPase activation. It is known that over-activation of the GTPases RhoA and Rac1 results in podocyte injury in mice [33–35]. Hence, we measured the activity levels of the GTPases Rho1 and Rac/Cdc42 in nephrocytes treated with DMSO or YODA to determine if these were downstream components of the pathway. We used Rok (a Rho1 sensor) and Pak (a sensor that reports on activity of Rac and Cdc42) sensors to visualize active Rho1 and Rac/Cdc42 in nephrocytes. Interestingly, analysis revealed a significant increase of active Rho1 levels while Rac/Cdc42 activation was significantly inhibited after YODA incubation **(Figure 3D,E)**.

Both, Ca^{++} and GTPases, have an impact of actin stress fibre formation. Therefore, we visualized the actin cytoskeleton by using flies expressing RFP-LifeAct in nephrocytes. Previous work revealed the presence of cortical stress fibres and actin clusters in *Drosophila* nephrocytes [36]. Although GTPase activation levels were altered by YODA treatment, no differences of actin stress fibre or cluster formation could be observed in comparison to control cells **(Figure 3F)**. The missing effect on actin stress fibre formation can be explained by the short incubation time with YODA (5 mins), which only induces and exhibits acute changes. Taken together, the pharmacological activation of Piezo by YODA confirmed Piezo expression in nephrocytes and delineated a mechanotransduction pathway, involving Ca^{++} signalling and GTPases.

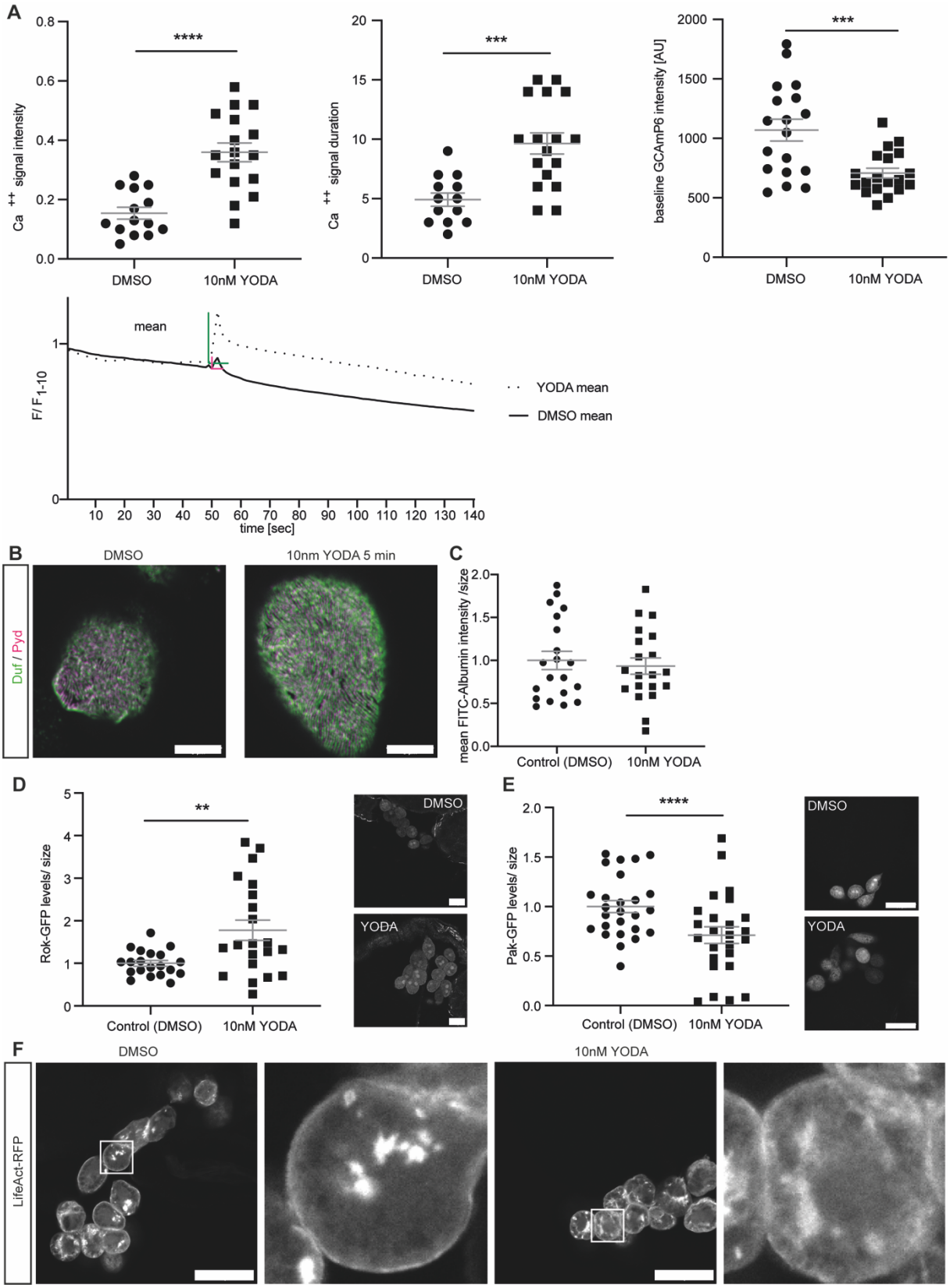

Figure 3: Pharmacological activation of Piezo causes Rho1 activation. A Ca^{++} signalling events were recorded after Piezo activation with YODA and after inducing shear stress after 60 sec. The immediate Ca^{++} wave observed after the induction of shear stress was significantly higher (peak intensity/amplitude; green and magenta line) and longer (peak duration; green and magenta line) in pericardial cells incubated with YODA when compared to controls (DMSO). Baseline Ca^{++} (GCAmP6m) levels were significantly reduced after 5 min incubation with YODA. Student's t-test: ***: $p < 0.001$, ****:

$p < 0.0001$. Visualisation of mean values of the DMSO and YODA treated nephrocytes show the differences in the amplitude after shear stress induction. The green and magenta lines depict the differences in amplitude height and width. w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*GCAmP6m*. **B** 5 min incubation with YODA did not reveal any morphological changes as depicted by immunofluorescence staining with a Duf and Pyd antibody. Scale bar = 5 μm. **C** The FITC-Albumin uptake assay did not reveal any differences between controls (DMSO) and YODA treated cells. FITC-Albumin intensity was normalized to nephrocyte size. **D** Quantification of active Rho1 levels (Rok-GFP) revealed a significant increase of Rho1-GTP levels after YODA incubation (scale bar = 25 μm), while **E** Rac/Cdc42-GTP levels were significantly decreased as depicted with Pal-GFP flies (scale bar = 50 μm). Student's t-test: *: $p < 0.05$; **: $p < 0.01$. w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*rok*-GFP (**D**); w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*pak*-GFP (**E**). **F** Visualisation of the actin cytoskeleton (LifeAct-RFP) did not reveal any changes upon YODA treatment. Scale bar = 50 μm. w; *sns*-Gal4/+;UAS-*dicer2*/UAS-LifeACT-RFP.

Loss of Piezo only resulted in a mild filtration defect

After the expression of Piezo in nephrocytes was confirmed, we wanted to know if Piezo is important for nephrocyte morphology and function. To address this question, we examined nephrocyte diaphragm morphology and filtration function in animals completely lacking Piezo ($Piezo^{KO}$). Loss of Piezo did not result in any changes of the slit diaphragm integrity as assessed by immunofluorescence **(Figure 4A)**. FITC Albumin uptake assays revealed a significant increase of uptake after depletion of Piezo (whole body knockout and nephrocyte-specific depletion) **(Figure 4B,C)**. However, the FITC Albumin assay cannot distinguish between uptake, filtration and endocytosis. To assess the effect of Piezo depletion on filtration function in more detail, we performed the $AgNO_3$ toxin assay. During larval development nephrocytes are important to clear the haemolymph from toxins and waste products to ensure normal development. By feeding larvae $AgNO_3$ containing yeast, the toxin will accumulate in the haemolypmh and needs to be cleared by nephrocytes to prevent a developmental delay (pupation delay). Impaired filtration function and endocytosis will result in less clearance efficiency and therefore cause a developmental delay of larvae, which can be used as an indirect way to assess filtration function. As the $Piezo^{KO}$ lesion is in a w^{1118} background ($Piezo^{KO}$ was backcrossed into w^{1118} for six generations prior to

experiments), we used this strain as a control for the Piezo^{ko}. Interestingly, the w^{1118} strain presented with a delayed pupation, which was similar to the Piezo^{ko}, suggesting that the filtration defect is not caused by depletion of Piezo, but rather due to the genetic background **(Figure 4D)**. To further confirm, that loss of Piezo does not influence filtration function and therefore pupation, we also used a Piezo-RNAi fly strain to deplete Piezo only in nephrocytes and could show, that a knockdown of Piezo also did not reveal any differences in pupation when compared to control flies **(Figure 4E)**.

We previously showed an impact of pharmacological Piezo activation on Ca^{++} signalling and GTPase activation and wanted to know whether depletion of Piezo results in defects in Ca^{++} signalling and altered GTPase activity levels as well. To do so we utilized Piezo-specific RNAi in combination with different sensor fly strains. Combining the GCaMP6medium sensor fly strain with the Piezo RNAi resulted in flies which express the sensor and deplete Piezo in nephrocytes simultaneously. Recording of the Ca^{++} signals did show a significant decrease of the Ca^{++} signal intensity (amplitude) upon shear stress after depletion of Piezo, confirming again its activity in nephrocytes and its involvement in Ca^{++} signalling in these cells **(Figure 5A)**. Quantification of the signal duration (peak duration) and the Ca^{++} (GCAmP6) baseline levels, did not reveal any significant differences **(Figure 5A)**. Again, we investigated GTPase activity levels in Piezo knockdown nephrocytes, as Ca^{++} signalling was significantly impaired and Ca^{++} is known to be involved in GTPase activation. Interestingly, loss of Piezo did not result in changes of active Rho1 and Rac/Cdc42 levels **(Figure 5B,C)**.

Our data shows that loss of Piezo only results in a mild filtration phenotype, but does not impact on GTPase activation.

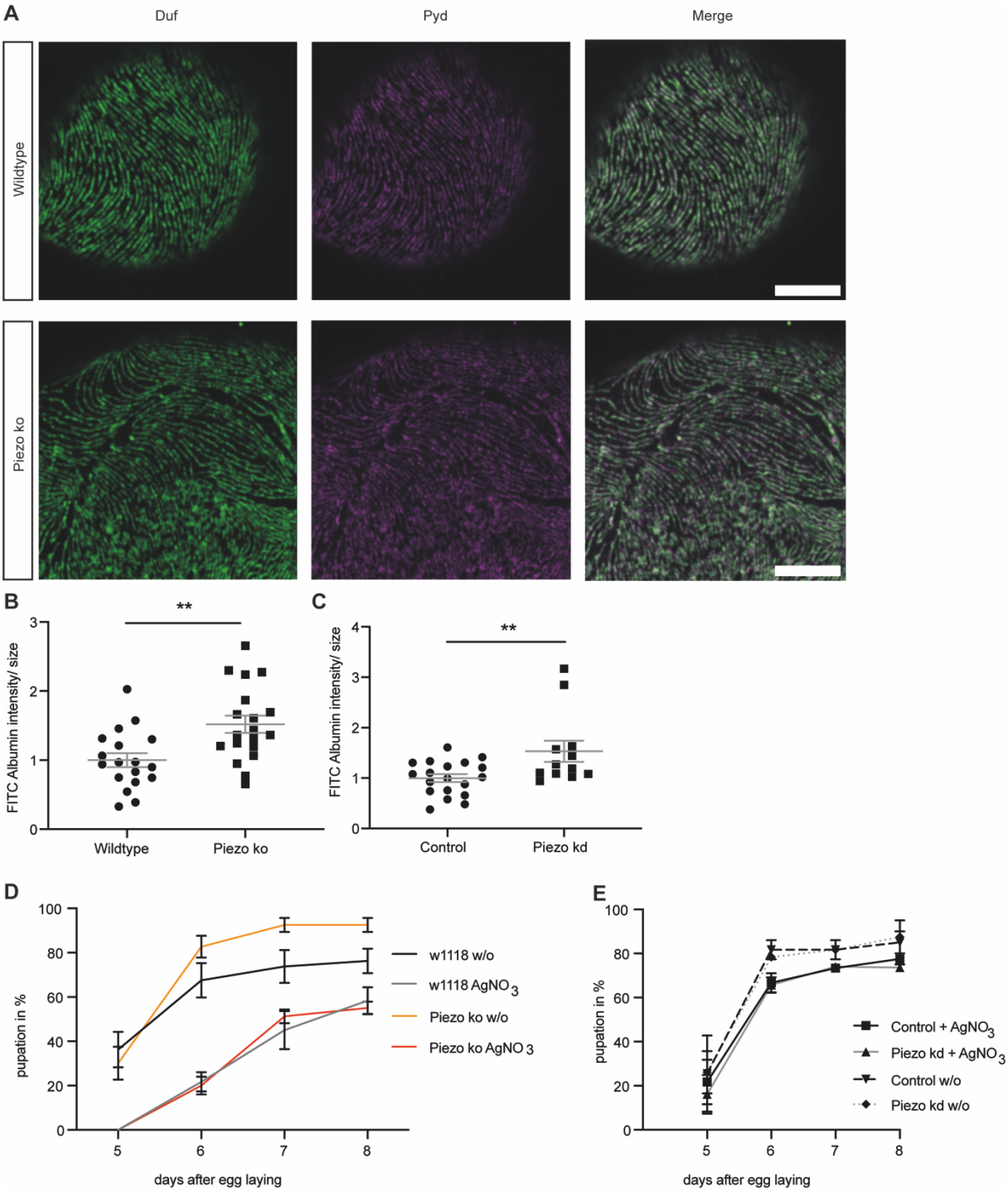

Figure 4: Loss of Piezo caused a mild filtration phenotype. A Immunofluorescence staining with a Duf and Pyd antibody did not reveal any morphological changes in Piezo^{ko} (w;+;*piezo*^{ko}) nephrocytes in comparison to control cells (wildtype w^{1118}). Scale bar 5 = µm. **B,C** FITC Albumin uptake assays revealed a filtration defect with an increased FITC-Albumin uptake in Piezo^{ko} and Piezo knockdown nephrocytes. Student's t-test: **: $p < 0.01$. Control: w;*sns*-Gal4/+;UAS-*dicer2*/+; Piezo kd: w;*sns*-Gal4/ UAS-*piezo*-RNAi;UAS-*dicer2*/+. **D** $AgNO_3$ toxin assays did not revealed a filtration defect, as Piezo^{ko} flies showed a similar delay in pupation when compared to controls (wildtype w1118). **E** AgNO3 toxin assay using Piezo RNAi also did not reveal any differences in pupation behaviour, when compared to control flies.

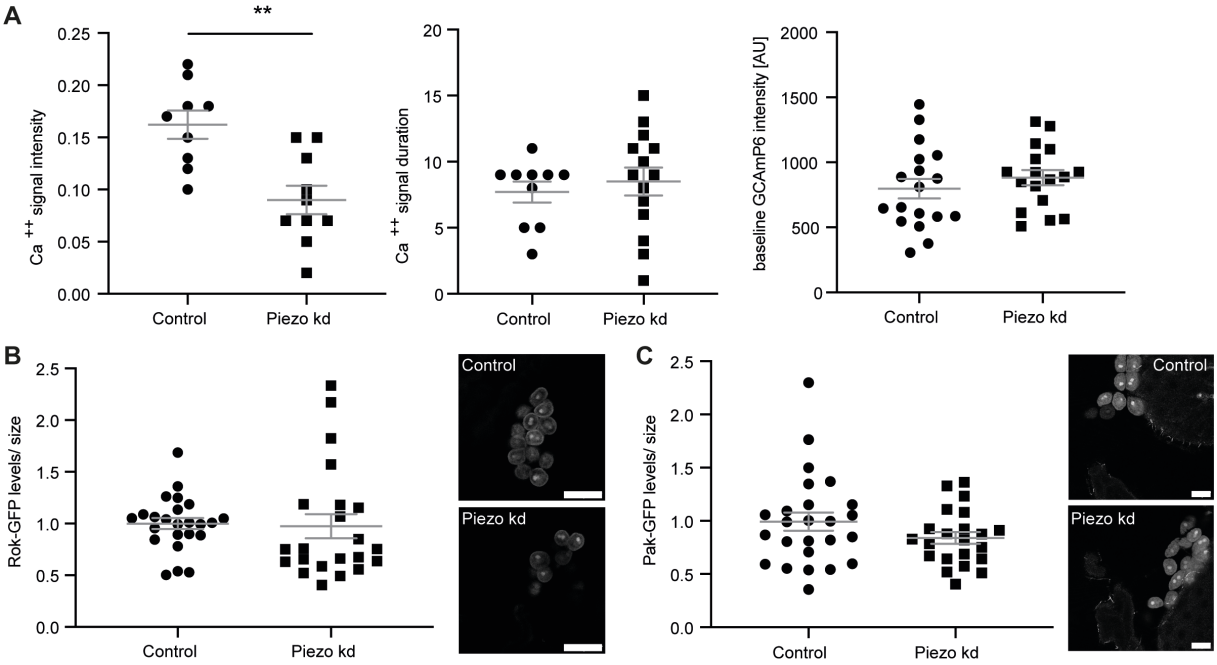

Figure 5: Piezo knockdown resulted in impaired Ca^{++} signalling, but did not affect GTPase activation. A Ca^{++} imaging and quantification of the GCAmP6 signal did reveal a significant decrease in signal intensity after depletion of Piezo. Signal duration and baseline Ca^{++} (GCAmP6) levels were not changed upon loss of Piezo. Control: w; *sns*-Gal4/+;UAS-*GCAmP6m*/+; Piezo kd: w; *sns*-Gal4/UAS-*piezo*-RNAi;UAS-*GCAmP6m*/+. Student's t-test: **: $p < 0.01$. **B** Quantification of the Rho1 (Rok-GFP) and **C** Rac/Cdc42 (Pak-GFP) activity levels in Piezo depleted cells did not reveal any differences to control cells. (scale bar = 50μm (**B**) = 25μm(**C**)). Control: w; *sns*-Gal4/+;UAS-*rok-GFP*/+; Piezo kd: w; *sns*-Gal4/UAS-*piezo*-RNAi;UAS-*rok-GFP*/+ (**B**); Control: w; *sns*-Gal4/+;UAS-*pak-GFP*/+; Piezo kd: w; *sns*-Gal4/UAS-*piezo*-RNAi;UAS-*pak-GFP*/+ (**C**).

Elevated Piezo levels caused a severe filtration phenotype, significantly increased Rho1-GTP levels and actin stress fibre formation

Piezo activation with YODA confirmed the presence of Piezo in nephrocytes and its involvement in a mechanotransduction pathway including Ca^{++} signalling and GTPases. Depletion of Piezo by knockout and knockdown revealed a mild filtration defect, but no impact on GTPase activation. As pharmacological activation of Piezo resulted in increased active Rho1 levels, which are described to be detrimental for podocyte biology [33], and loss of Piezo only caused a mild phenotype, we further investigated whether an overexpression of the channel results in a severe nephrocyte phenotype. In contrast to the acute intervention by YODA application, overexpression

of Piezo in nephrocytes is a chronic intervention, which gives the cells days to manifest a phenotype.

First, we unravelled the effect on Ca^{++} signalling. Again, we induced shear stress after 60 secs by pipetting buffer on the tissue (red arrow) and observed an immediate Ca^{++} peak in controls **(Figure 6A)**. Imaging Piezo overexpression cells revealed a constant oscillating Ca^{++} signal and no additional peak when adding buffer (red arrow) **(Figure 6B)**. This constant, force-independent oscillations seem to be mediated by a significant increase of Piezo channels expressed in nephrocytes, resulting in more Ca^{++} permeable channels. Whether the increased density of channels at the nephrocyte diaphragm also causes an opening of the channels remains unclear, but our data shows, that even without shear stress by adding buffer, we observe an oscillating Ca^{++} signal pattern **(Figure 6A,B)**.

We then analysed the impact of increased Piezo levels on nephrocyte morphology and function. The UAS-Gal4 system we are using to express Piezo in nephrocytes exclusively is temperature-sensitive, which enables us to increase expression levels with temperature. We used two different temperatures (medium: 25°C and high: 28°C) for Piezo overexpression. Of note, all other experiments (pharmacological activation and Piezo depletion) were performed at 25°C.

Our data show, that morphology was not impacted by Piezo overexpression at 28°C (high levels) **(Figure 6C).** FITC Albumin uptake was significantly increased in cells expressing high levels (28°C) when compared to control cells **(Figure 6D)**. Interestingly, the $AgNO_3$ toxin assay revealed a severe filtration defect already at 25°C, with a significant delay of pupation detectable from day 5 **(Figure 6E)**.

Next, we investigated the impact of increased Piezo levels on GTPase activation. Overexpression of Piezo resulted in significantly increased Rho1-GTP levels, while active Rac/Cdc42 levels were not altered **(Figure 6F,G)**. We observed the same effect

for active Rho1 levels, when treating cells with YODA. As activation of Rho1 was described to cause actin stress fibre formation in podocytes [37], we again visualized the actin cytoskeleton with LifeAct-RFP. A previous study investigated the actin cytoskeleton in nephrocytes and could show the presence of cortical stress fibres and actin clusters in the cells [36]. Interestingly and in line with the activation of Rho1, overexpression of Piezo caused a significant increase of both, cortical actin fibres and actin clusters, when compared to controls **(Figure 6H)**. This finding is in contrast to the actue Piezo activation by YODA application and suggests a more severe phenotype when Piezo levels are permanently increased and active. Moreover, the data show that actin stress fibre formation is a slow process. Our data again confirms that Piezo is involved in regulating Ca^{++} signalling and Rho1 activation and also shows that the actin cytoskeleton is part of the adaptive behaviour in response to the mechanotransduction pathway as well.

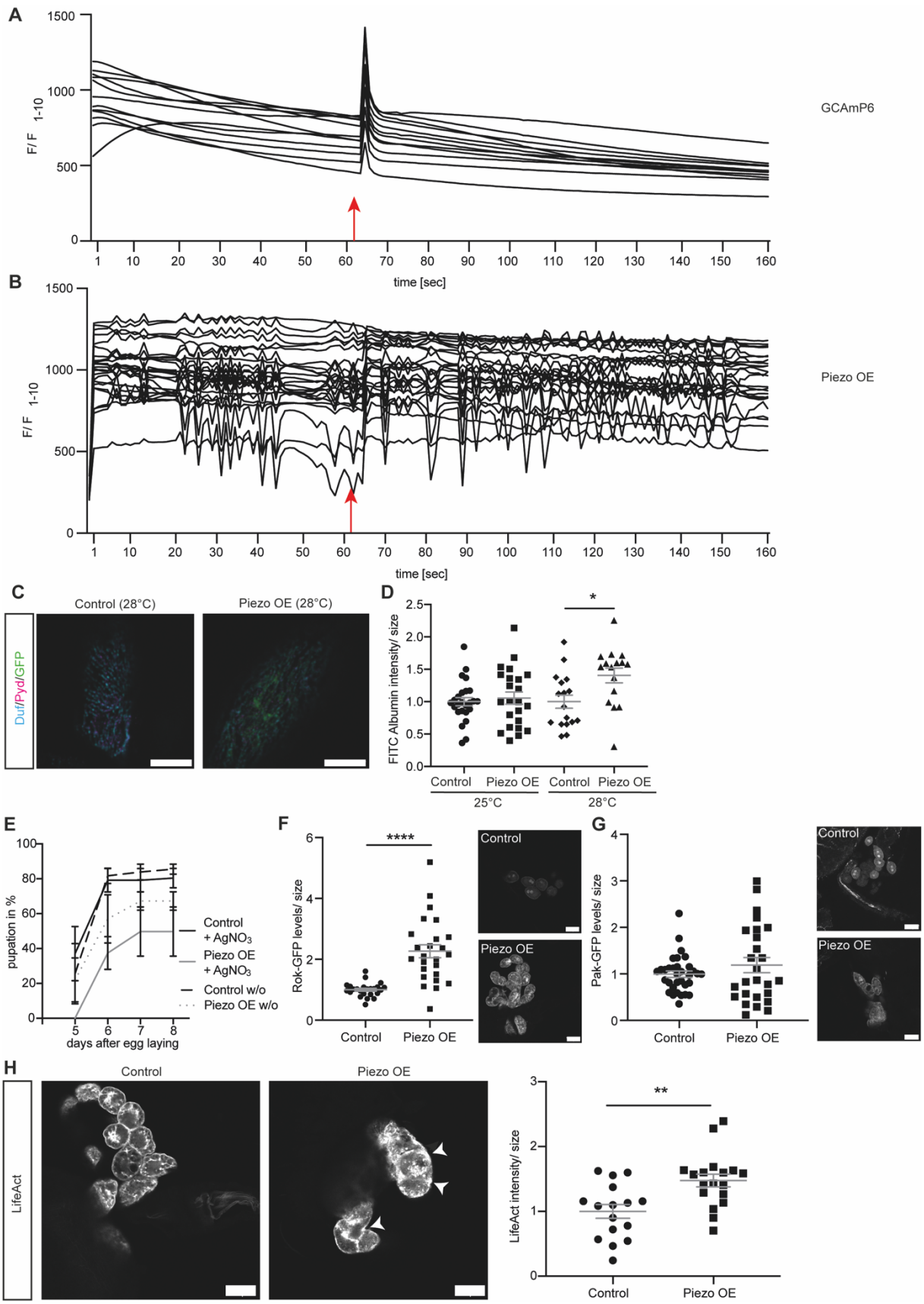

Figure 6: Elevated Piezo levels cause a severe filtration phenotype and result in elevated Rho1-GTP levels and more actin stress fibers. A Ca^{++} imaging of GCAmP6 expressing nephrocytes revealed an immediate increase of the GCAmP signal/amplitude after inducing shear stress by adding buffer (red arrow). Depicted is one animal and one curve is one nephrocyte. Images were taken every

second for 150secs. For analysis and quantification values were normalized to F_{1-10}. Control: w; *sns*-Gal4/+;UAS-*GCAmP6m*/+. **B** Ca^{++} imaging of nephrocytes expressing Piezo and GCAmP6 simultaneously. The elevated Piezo levels resulted in a constant oscillating GCAmP6 intensity pattern. Inducing shear stress by adding buffer did not result in an immediate increase of the intensity as observed for controls. Depicted is one animal and one curve is one nephrocyte. Images were taken every second for 150secs. For analysis and quantification values were normalized to F_{1-10}. Piezo OE: w; *sns*-Gal4/+;UAS-*GCAmP6m*/UAS-*piezo*-GFP. **C** Immunofluorescence staining with a Duf (cyan) and Pyd (magenta) antibody did not reveal any morphological changes in nephrocytes expressing Piezo (green). Flies were kept at 28°C to increase protein expression levels. Scale bar = 25μm. Control: w; *sns*-Gal4/+;UAS-*dicer2*/+. Piezo OE: w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*piezo*-GFP. **D** FITC Albumin assays revealed a significantly increased FITC Albumin uptake in nephrocytes expressing Piezo at 28°C, while no filtration defect was observed at 25°C. Student's t-test: *: $p < 0.05$. **E** AgNO3 toxin assays confirmed a severe filtration defect, as larvae expressing Piezo in nephrocytes present with a significantly delayed pupation behaviour already at 25°C. 2way-ANOVA with Tukey's multiple comparison test: *: $p < 0.05$. **F** Piezo expression caused a significant increase of active Rho1 levels in nephrocytes. Student's t-test: ****: $p < 0.0001$. Scale bar = 25μm. Control: w; *sns*-Gal4/+;UAS-*rok*-GFP/+. Piezo OE: w; *sns*-Gal4/+;UAS-*rok*-GFP/UAS-*piezo*-GFP. **G** Rac-GTP levels were not altered upon expression of Piezo in nephrocytes. Scale bar = 25μm. Control: w; *sns*-Gal4/+;UAS-*pak*-GFP/+. Piezo OE: w; *sns*-Gal4/+;UAS-*pak*-GFP/UAS-*piezo*-GFP. **H** Visualisation of the actin cytoskeleton revealed an increase of cortical actin stress fibers (arrow heads) and actin clusters in nephrocytes expressing Piezo. Quantification was done for the whole cell. Student's t-test: **: $p < 0.01$. Scale bar = 25μm. Control: w; *sns*-Gal4/+;UAS-*LifeACT*-RFP/+. Piezo OE: w; *sns*-Gal4/+;UAS-*LifeACT*-RFP/UAS-*piezo*-GFP.

Pharmacological inhibition with tarantula toxin reversed the elevated Rho1-GTP levels in Piezo overexpressing cells

Overexpression of Piezo resulted in a severe nephrocyte phenotype including filtration disturbances, significantly increased Rho1-GTP levels and more actin stress fibres and clusters. Therefore, we tested whether pharmacological inhibition of the channel could reverse the phenotype and be beneficial for nephrocyte function. To do so, we used tarantula toxin (GSMTx4), which integrates into the cell membrane and blocks the opening of the channel upon mechanical stretch [38] .

Acute inhibition of the mechanosensitive channel did not result in a morphology or filtration function phenotype **(Figure 7A,B)**. GTPases assessment and analysis of the actin cytoskeleton also did not reveal significant differences in comparison to control cells **(Figure 7C,D,E,F)**. Overexpression of Piezo caused a highly significant increase of active Rho1 levels, hence we treated the Piezo overexpression nephrocytes with tarantula toxin and quantified Rho1-GTP levels. 5 min treatment with

the toxin significantly reduced the Rho1-GTP levels and resulted in a rescue **(Figure 7G).** Taken together, our data demonstrates that tarantula toxin can be used to reverse the overactive Piezo effect on GTPase activation. A treatment with the toxin did not reveal any pathological effects on nephrocyte morphology and function and can therefore be used as an intervention.

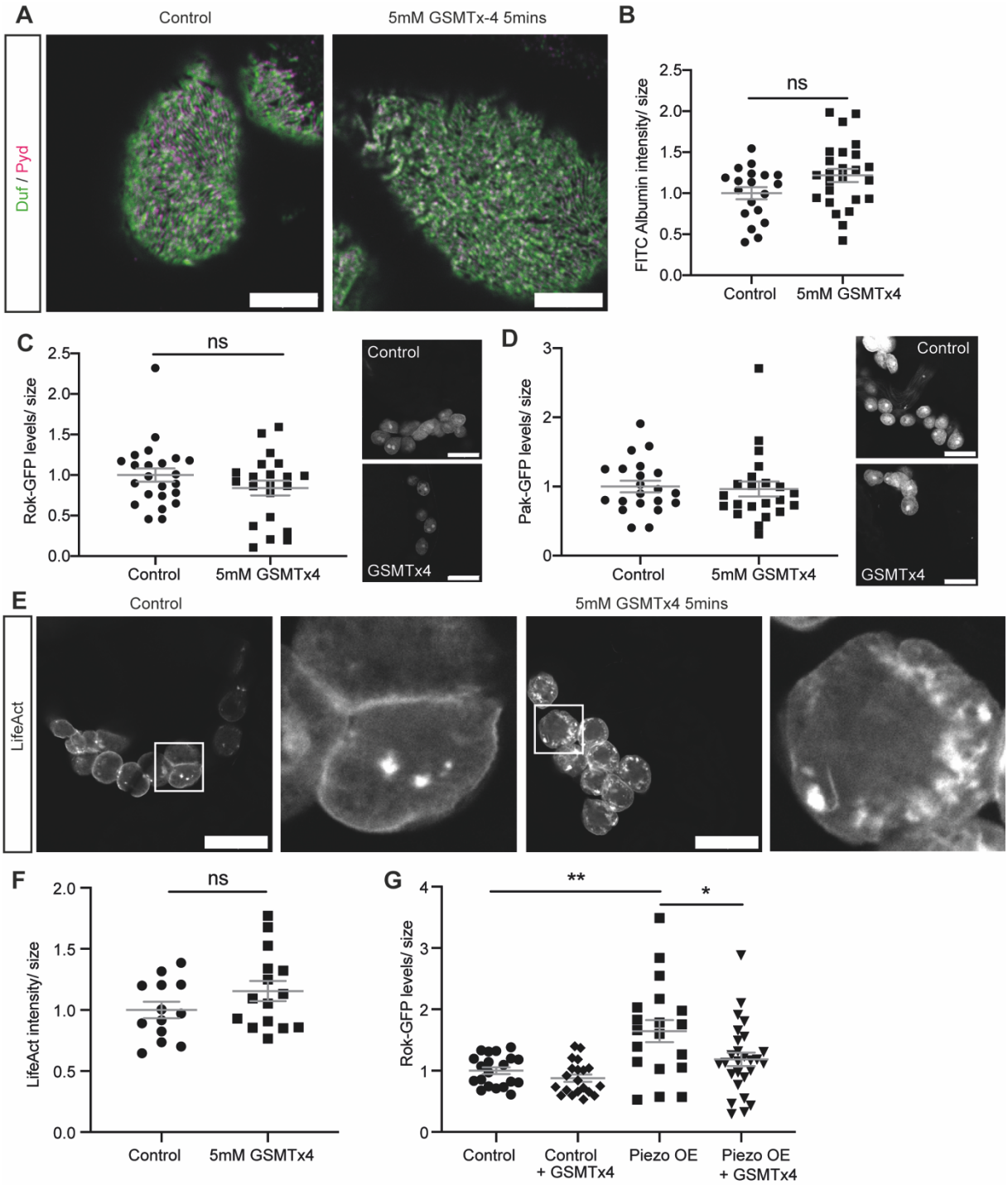

Figure 7: GSMTx4 treatment did not result in a nephrocyte phenotype and reversed elevated Rho1 activity levels in Piezo overexpression cells. A Immunofluorescence staining with Duf and Pyd antibodies did not reveal any morphological changes after incubation with 5mM GSMTx4 for 5 min. Scale bar = 5 μm. **B** FITC Albumin uptake assays did not reveal any differences between controls and nephrocytes incubated with GSMTx4 for 5 min. **C,D** Quantification of GTPase activity levels did not reveal any changes in Rho1-GTP (Rok-GFP) or Rac/Cdc42-GTP (Pak-GFP) levels. Scale bar = 50 μm. Rok-GFP: w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*rok*-GFP (**C**). Pak-GFP: w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*pak*-GFP (**D**). **E,F** Visualization of the actin cytoskeleton did not show a significant increase of actin clusters after incubation of nephrocytes with GSMTx4 for 5 min. Scale bar = 50 μm. w; *sns*-Gal4/+;UAS-*dicer2*/UAS-*LifeACT*-RFP. **G** Rho1 activity levels were significantly reduced in Piezo overexpressing cells (w; *sns*-Gal4/+; UAS-*rok*-GFP/UAS-*Piezo*-GFP) after 5 min treatment with GSMTx4. Comparison with control cells revealed a very good rescue, as Rho1-GTP levels were not significantly higher in Piezo overexpressing cells treated with GSMTx4 when compared to controls (w; *sns*-Gal4/+; UAS-*rok*-GFP/+). 1-Way ANOVA with Tukey's multiple comparison test: *: $p < 0.05$; **: $p < 0.01$.

Discussion

Mechanotransducers play a crucial role in podocyte biology and within this study, we were able to delineate the physiological role of the mechano-sensitive ion channel Piezo in nephrocytes for the first time. Here, we provide evidence that Piezo is expressed at the nephrocyte diaphragm and co-localizes with Duf, the NEPH homolog in the fly, suggesting Piezo to be a novel component of the diaphragm protein complex. Our data shows that Piezo is involved in a mechanotransduction pathway, which includes Ca^{++} signalling, Rho1 activation and subsequently actin stress fibre and actin cluster formation in nephrocytes. An increase of protein levels therefore results in filtration disturbances, a severe nephrocyte phenotype.

We also confirmed that nephrocytes respond to shear stress by opening mechanosensitive channels resulting in Ca^{++} influx. This response is significantly reduced upon injury by depleting Duf (dNEPH) or Sns (dNephrin). Loss of either of those nephrocyte diaphragm proteins results in a severe morphological phenotype, with loss of the lacunae structure and degradation of the nephrocyte diaphragm [26,27]. Whether mechanosensitive channels are degraded as well or misslocalized remains an open question.

The tight regulation of GTPases is essential to maintain podocyte health and survival. Previous studies revealed a pathological effect of overactive RhoA and Rac1 in mice. This includes albuminuria as well as foot process effacement and the activation of both is associated with FSGS and minimal change disease [33–35]. In line with this, the overactivation of Rho1 in nephrocytes also resulted in a severe nephrocyte phenotype with morphological and functional disturbances [39]. Mechanosensors, such as TRP channels, also have been described to regulate GTPase activation in podocytes [40]. One downstream target of GTPases is the actin cytoskeleton, which plays an essential role during podocyte injury and rearrangement processes [41]. Here, we identified increased activation of Rho1 after YODA treatment and overexpression of Piezo. In addition, YODA treatment caused decreased Rac1 activity levels but did not change the actin cytoskeleton. YODA treatment was only performed for 5 mins, hence rearrangement processes of the actin cytoskeleton might not have happened. The impact of over-active Rho1 on the actin cytoskeleton in nephrocytes remains unknown, but the expression of an constitutively active Rho1 variant resulted in a severe nephrocyte phenotype, including morphological changes and filtration disturbances [39]. In cultured podocytes, the expression of over-active RhoA (in *Drosophila* Rho1) caused a significant increase of actin stress fibers [37], a similar effect to what we observed in the Piezo overexpression nephrocytes.

Our findings, that GTPase activation and subsequent actin stress fibre formation are crucial aspects of the podocytes response to injury, are supported by a previous study investigating the effect of Bis-T-23 in podocytes. Bis-T-23 promotes actin-dependent dynamin oligomerization and stabilizes the actin cytoskeleton during podocyte injury [42]. In detail, several injury models which caused actin-cytoskeleton rearrangement could be at least partially rescued by Bis-T-23 treatment, again emphasising the importance of the actin-cytoskeleton and upstream regulators such as GTPases [42].

Due to their position podocytes are constantly facing changing biomechanical forces such as hydrostatic pressure and shear stress. Therefore, it is not surprising that recent studies identified mechanosensors to play a crucial role for podocyte biology. Among them are Talin [17], Vinculin [43] and Filamin [8,9,18]. The mechano-sensitive ion channel Piezo has recently been found to be expressed in mouse podocytes, but its exact localisation pattern and its functional role in these cells is not known [19]. Interestingly, Piezo levels were not altered in a hypertension rat model nor in a podocyte injury mouse model (Doxorubicin) [8,44]. Although total Piezo levels were not changed overactive variants might result in pathological effects and glomerular disease phenotypes. A recent publication identified a gain-of-function mutation present in a third of the African population, which results in the resistance to plasmodium infection due to dehydrated red blood cells [45]. Clinical evaluation of individuals carrying this allele will be of greatest interest, in particular in regard to kidney function.

Assuming a correlation between over-active Piezo variants and a glomerular disease phenotype a treatment to block Piezo activation could be beneficial for patients. Our data showed that the treatment with tarantula toxin (GSMTx4) was able to reverse the increased Rho1-GTP levels. Interestingly, GSMTx4 is currently used in clinical trials as a novel treatment for muscle dystrophy (https://laforcedmd.com/tarantula-venom-dmd/) and would therefore be an ideal candidate for patients suffering from Piezo over-activation induced glomerular disease. Of note, GSMTx4 is not a specific Piezo inhibitor and can affect other mechano-sensitive channels as well. Also, systemic effects of GSMTx4 application cannot be ruled out at the moment. Nonetheless, GSMTx4 seems to be a promising novel therapeutic.

Taken together, here we provide evidence that the mechano-sensitive ion channel Piezo is localizing to the nephrocyte diaphragm, needs to be tightly controlled

to maintain nephrocyte morphology and function and regulates Rho1 activity levels and the formation of actin stress fibers via Ca^{++} influx.

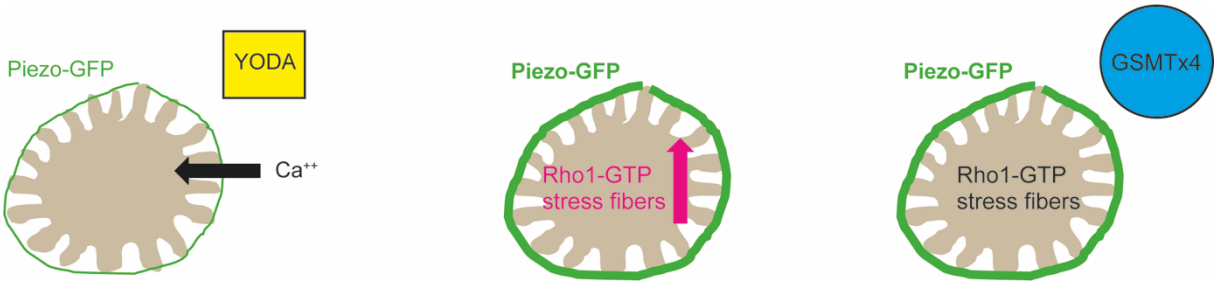

Figure 8: Schematic of Piezo expression and function in *Drosophila* nephrocytes. Piezo localizes to the nephrocyte diaphragm and co-locailzes with Duf and Pyd. Activation of the channel with its agonist YODA results in an increased Ca^{++} signal and activation of Rho1 (left panel). Elevated Piezo levels have a pathological effect on nephrocyte biology and cause a severe filtration defect. In line with the YODA effects, elevated Piezo levels also result in activation of Rho1 and an increased formation of cortical actin stress fibers and actin clusters (middle panel). Treatment with the antagonist GSMTx4, tarantula toxin, can reverse the elevated Rho1-GTP levels upon increased Piezo levels, exhibiting a potential as a future therapeutic (right panel).

Author contribution

S.K. conceived the study, performed experiments, analysed the data, made the figures and drafted the paper, B.D. revised the study critically for important intellectual content, S.K. and B.D. revised the paper; both authors approved the final version of the manuscript.

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Conflict of Interest

None. The authors have nothing to disclose.

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