Polycystin-1 Regulates Actomyosin Contraction and the Cellular Response to Extracellular Stiffness

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Polycystin-1 (PC-1) and 2 (PC-2) are the products of the PKD1 and PKD2 genes, which are mutated in Autosomal Dominant Polycystic Kidney Disease (ADPKD). They form a receptor/channel complex that has been suggested to function as a mechanosensor, possibly activated by ciliary bending in the renal tubule, and resulting in calcium influx. This model has recently been challenged, leaving the question as to which mechanical stimuli activate the polycystins still open. Here, we used a SILAC/Mass-Spec approach to identify intracellular binding partners of tagged-endogenous PC-1 whereby we detected a class of interactors mediating regulation of cellular actomyosin contraction. Accordingly, using gain and loss-of-function cellular systems we found that PC-1 negatively regulates cellular contraction and YAP activation in response to extracellular stiffness. Thus, PC-1 enables cells to sense the rigidity of the extracellular milieu and to respond appropriately. Of note, in an orthologous murine model of PKD we found evidence of increased actomyosin contraction, leading to enhanced YAP nuclear translocation and transcriptional activity. Finally, we show that inhibition of ROCK-dependent actomyosin contraction by Fasudil reversed YAP activation and significantly improved disease progression, in line with recent studies. Our data suggest a possible direct role of PC-1 as a mechanosensor of extracellular stiffness.

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a relatively common monogenic disorder, affecting approximately 1.2000 individuals8,9. The disease is due to mutation in either the PKD1 or the PKD2 genes, encoding for two large membrane proteins, Polycystin-1 (PC-1) and Polycystin-2 (PC-2), respectively. PC-1 is a transmembrane protein with a long extracellular domain, 11 transmembrane domains and a short intracellular C-tail2–4. PC-1 C-terminal interacts through a coiled-coil domain with the C-terminal of PC-2, a cation channel of the TRPP family, to form the PC-1/PC-2 complex, likely with an intrinsic channel activity5,6. The PC-1/PC-2 complex localizes to cilia, as well as to sites of cell-cell/matrix interaction2,3,7–9. Although the PC-1/PC-2 complex has been described to play a role in several molecular pathways, its function remains to be understood2. One key activity of the polycystins receptor/channel complex has been ascribed to the capability of PC-1 to regulate key morphogenetic programs such as renal tubular morphogenesis both in vitro10,11 and in vivo12,13 as well as lymphatics development14. Importantly, overexpression of PC-1 was reported to drive a program of cell migration15,16 and front-rear polarity via its role in regulating focal adhesions17 and adherent junctions15 turnover as well as by regulating the actin cytoskeleton and the function of a Par3/aPKC complex12,13,18. The molecular mechanisms involved in this specific function of PC-1, however, remain largely elusive. Likewise, the mechanism of activation of this receptor/channel complex remains unclear. Chemical stimuli such as Extracellular Matrix (ECM) components and Wnt proteins have been shown to interact with PC-1 and to mediate its activation18, highlighting the possibility of this receptor being regulated by specific chemical ligands. In addition to this property, a possible role as a mechanosensor for PC-1 has been postulated on the basis of the fact that the predicted structure of PC-1 is very similar to proteins with structural and mechanical roles19–23. Specifically, the PC-1 extracellular region, rich in extensible Ig-like domains, shows elastic properties suggesting its role as a potential mechanosensor22. Furthermore, PC-1 has been shown to sense and transduce mechanical forces into biochemical signals24–30. In particular, based on its localization to cilia, PC-1 has been long believed to be the mechanosensor enabling renal epithelial cells to respond to the bending of cilia caused by urine flow resulting in calcium influx24. However, recent studies have questioned this PC-1 and PC-2 function31. Finally, evidence of PC-1 classification as

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a mechanosensor are based on its interaction with both components of the ECM and the cytoskeleton via intermediate filaments. Of note, it has been shown that inactivation of integrin β1 in collecting ducts, a known positive mechanosensor of extracellular stiffness, led to inhibition of Pkd1-dependent cystogenesis in vivo.

However, while the role of PC-1 extracellular domain has long been postulated to be that of a mechanical sensor, the precise signal that activates the mechanical properties of the receptor remains unknown. Polycystins are expressed in tissues other than kidney, such as bone where matrix stiffness has an important impact. In line with this, a recent study suggests that PC-1 has a role in regulating osteoblastogenesis and adipogenesis through interaction with the transcriptional coactivator with PDZ-binding motif (TAZ). Mechanical forces, such as ECM stiffness activate the PC-1/PC-2 complex leading to induction of PC-1. As a control, we used MEFs derived from the C-terminus of PC-1 to allow the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody. As a control, we used MEFs derived from the C-terminus of PC-1 to allow the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody. As a control, we used MEFs derived from the C-terminus of PC-1 to allow the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody. As a control, we used MEFs derived from the C-terminus of PC-1 to allow the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody. As a control, we used MEFs derived from the C-terminus of PC-1 to allow the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody.

Materials and Methods

Results

Generation of the first PC-1 interactome reveals its central role in regulation of the actomyosin machinery. PC-1 has been proposed to play a role in multiple signaling pathways but its main physiological function is far from being understood. To gather an overview of a possible central function of PC-1, we set out to generate the interactome of the endogenous protein under stringent conditions. We isolated mouse embryonic fibroblasts (MEFs) from a Pkd1HA/HA mouse model, in which an HA tag has been inserted in-frame in the last exon of the Pkd1 gene resulting in a fully functional endogenous PC-1 protein. The presence of the HA tag at the C-terminus of PC-1 allowed the immunoprecipitation (IP) of the endogenous protein using commercial beads covalently linked to an anti-HA antibody. As a control, we used MEFs derived from Pkd1−/WT mice expressing equal levels of untagged PC-1. Two IP coupled to Mass Spectrometry approaches have been used, both based on SILAC (Stable Isotopes Labeling by Aminoacids in Cell culture). Briefly, Pkd1HA/HA and Pkd1−/WT MEFs were grown in the presence of amino acids labeled with different isotopes (heavy-13C and light-12C, respectively) and, upon HA IP, samples were mixed and analyzed together (Fig. 1a,b). In the first approach, named SILAC1, we run the immunoprecipitated proteins on the gel and we performed in-gel proteins digestion followed by liquid chromatography mass spectrometry; in the second approach, named SILAC2, the digestion of the proteins was performed directly on the beads. While soluble proteins are better digested in solution, membrane proteins, such as PC-1, are better digested if completely denatured in the gel; therefore, a combination of the above strategies is the best choice to increase interactome coverage as much as possible. For each method two technical replicates were performed. Globally, we identified 316 proteins, 30 of them significantly enriched in the IP versus the control (Fig. 1c,d and Supplementary Table 1), including the bait itself PC-1. A scatter plot of the interactors with their heavy/light ratio is reported (Fig. 1c) together with the functional protein association networks, based on STRING database (Fig. 1e). When inspecting the list of interactors, we were pleased to see that a few known interactors were represented in the list, including the Goi subunit of heterotrimeric G proteins, previously demonstrated to interact with PC-1. Much to our surprise the list of interactors and the STRING analysis pointed to a single network of functional regulators of vascular smooth muscle contraction, regulation of actin cytoskeleton machinery and focal adhesions (Fig. 1e).

PC-1 regulates actomyosin contraction. Our Mass Spectrometry data indicate that several interactors of PC-1 are proteins involved in the regulation of the actomyosin machinery. Therefore, we investigated the phosphorylation state of myosin light chain (pMLC), the main regulator of actomyosin contraction, in Pkd1 gain and loss of function cellular models. We found that the levels of pMLC are decreased in Madine Darby Canine Kidney (MDCK) cells compared to controls (Fig. 2a and Supplementary Fig. 1). Conversely, MEFs isolated from Pkd1−/− mice showed an increased phosphorylation state of pMLC (Fig. 2b and Supplementary Fig. 1). These results suggest that PC-1 is a negative regulator of the phosphorylation state of MLC. We therefore tested whether PC-1 can effectively regulate the contractility of cells performing a collagen contraction assay. In this assay, cells are plated on top of collagen discs that are allowed to polymerize in multwell. Collagen discs are then mechanically detached from the border of the wells, and followed over time. The contractility of cells causes a progressive reduction of the area covered by the discs, thus disc-area is a direct measure of contractility and it is inversely correlated with the capability of cells to contract. Collagen discs in the absence of cells were used as controls to define a complete absence of contraction. Consistent with their pMLC levels, MDCK cells retracted collagen discs to a lesser extent, compared to control MDCK cells.
cells (Fig. 2c,d). On the contrary we observed a fast and remarkable contraction capability of Pkd1−/− MEFS (Fig. 2c,d) and PC-1 silenced Inner Medullary Collecting Duct (IMCDshPkd1) cells, compared to their controls (Fig. 2e). Thus, the expression levels of PC-1 inversely correlate with the pMLC levels and the capability of cells to contract. Next, we wondered which molecules upstream of the pMLC could play a role in the Pkd1−/− cells. The phosphorylation levels of MLC are regulated at multiple levels, and in particular Rho kinase (ROCK) and Myosin Light Chain Kinase (MLCK) can both enhance the levels of pMLC. In addition, ROCK inhibits the phosphatase responsible for dephosphorylation of MLC (MYPT1) ultimately enhancing the contraction capability of myosin (Fig. 3a). Notably, MYPT1 appears among the direct interactors of PC-1 (PPP1r12a gene in Fig. 1d). Interestingly, both inhibitors of ROCK (Y27632) and MLCK (ML-7) were able to decrease the phosphorylation of MLC to control levels in Pkd1−/− cells (Fig. 3b and Supplementary Fig. 1). In line with this, both inhibitors prevented the enhanced contraction observed in the mutant cells (Fig. 3c).

PC-1 regulates the cellular response to extracellular stiffness. The actomyosin pathway plays a central role in the response of cells to mechanical stimuli such as ECM elasticity or cell density, and it has been shown to regulate the activity of the transcription factor YAP in response to mechanical stress. In particular, cells plated on stiff substrates or in sparse condition, show active nuclear localization of YAP that promotes cells proliferation and differentiation. On the contrary in cells cultured on soft substrates or in confluent condition, YAP is retained in the cytoplasm becoming functionally inactive (Fig. 4a). In the previous section we showed that PC-1 regulates myosin phosphorylation (Fig. 2a,b) and cell contractility (Fig. 2c,d,e). Furthermore, PC-1 structure suggests a possible role as a mechanosensor. We therefore wondered whether this receptor could be involved in sensing the rigidity of the extracellular environment. Given the central role of YAP localization in this type of response, we used YAP localization as a proximal marker to measure the cellular response to extracellular stiffness in the presence or absence of PC-1.

To test this hypothesis, we plated cells on hydrogels mimicking different stiffness of the matrix with an elasticity ranging from 0.5 KPa to 40 KPa as recently reported. Consistent with their low level of myosin phosphorylation, MDCKPKD1Zeo cells show an attenuated response to the increased stiffness displaying higher retention of YAP in the cytoplasm, compared to control MDCKZeo cells (Fig. 4b). We observed a comparable response when plating MDCKPKD1Zeo cells at low density since in epithelial cells the modulation of cell density recapitulates the response of the cell to the extracellular stiffness (Fig. 4c). As a consequence, expression of the YAP target genes

Figure 1. Polycystin-1 interactome study. (a) Proteomic workflow of the SILAC experiments for the identification of polycystin-1 interactome. (b) Example of HA IP showing PC-1. Full-length blot is presented in Supplementary Fig. 1. (c) All identified proteins were plotted according to their signal intensities and their heavy versus light ratio on logarithmic scales. (d) Histogram of the Log2 ratio H/L of the putative Pkd1 interactors in SILAC1 and SILAC2. (e) Functional protein association networks of the putative polycystin-1 interactors as reported in STRING database. In the network, links between proteins represent interaction data supporting the network. Additionally, enriched KEGG Biological Pathways (p < 0.05) are reported and the protein associated are color code circled.
Figure 2. Polycystin-1 regulates phosphorylation of myosin light chain and cell contractility. (a) PKD1 overexpressing epithelial MDCK cells (MDCK\(^{PKD1\text{Zeo}}\)) display a reduced phosphorylation level of MLC in activatory sites Thr18/Ser19 compared to control cells (MDCK\(^{Zeo}\)). Quantification of pMLC/MLC ratio in three independent experiments is presented in Supplementary Fig. 1. Bands were cropped from different parts of different gels. Full-length blots are presented in Supplementary Fig. 2. (b) Pkd1\(^{-/-}\) MEFs show hyperphosphorylation of MLC, compared to control Pkd1\(^{+/+}\) MEFs. Quantification of pMLC/MLC ratio in three independent experiments is presented in Supplementary Fig. 1. Bands were cropped from different parts of different gels. Full-length blots are presented in Supplementary Fig. 2. (c) Top: representative pictures at 24 h of three independent experiments of collagen contraction assay (each in triplicate) revealing a lower contraction of gels with MDCK\(^{PKD1\text{Zeo}}\) (clones C8/68 and G3/11) cells seeded compared to those with control MDCK\(^{Zeo}\) (clones F2 and F6) cells. Bottom: representative pictures at 24 h of three independent experiments of collagen contraction assay (each in triplicate) revealing a higher contraction of gels with Pkd1\(^{-/-}\) MEFs seeded, compared to those with Pkd1\(^{+/+}\) MEFs. As control (ctrl), gels without seeded cells were used to determine absence of contraction. (d) Quantification of the contraction assay shown in c. Cells contractility is estimated as the decrease of the ratio between the gel area at specific time points and the total well area. Top: MDCK\(^{PKD1\text{Zeo}}\) cells (clones C8/68 and G3/11) compared to control MDCK\(^{Zeo}\) cells (clones F2 and F6); bottom: Pkd1\(^{-/-}\) MEFs compared to Pkd1\(^{+/+}\) MEFs. Values are mean ± SD of three replicates. Statistical analysis: one-way ANOVA, followed by Bonferroni post-test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (e) Collagen contraction assay shows a higher contractility capacity of IMCD\(^{shPkd1}\) cells (clones C12 and C16) compared to control IMCD\(^{sh}\) cells (clones M3 and M4). Values are mean ± SD of three replicates. Statistical analysis: one-way ANOVA, followed by Bonferroni post-test; ****p < 0.0001. All experiments were performed at least three times and representative results are shown.
Ankrd1 and Cyr61 is down-regulated (Fig. 4d). This is in line with PC-1 localization at the site of cell-cell and cell-matrix junction other than at the primary cilium 3,4,9,50. According to a possible role of PC-1 in attenuating the response to extracellular stiffness, IMCD shPkd1 and Pkd1−/− MEFs fail to decrease YAP activation in soft conditions compared to controls (Fig. 4e,f), in line with a recent work29,41. Furthermore, we tested whether in epithelial cells silencing of Pkd1 conferred a lack of response to soft conditions generated by increased cell density40. Indeed, we found that IMCD shPkd1 show enhanced YAP nuclear translocation even when plated in confluent and hyper-confluent conditions (Fig. 4e).

The dysfunctional sensitivity of extracellular stiffness results in deregulated actomyosin in vivo. To address whether the role of PC-1 as a regulator of myosin activity is conserved in vivo, we examined pMLC levels in Pkd1 kidney-specific conditional mutants (Pkd1flox/−:KspCre), displaying a cystic phenotype. Consistent with data in cells, we found increased phosphorylation of MLC in cystic kidneys at both P5 and P12, compared to controls (Fig. 5a and Supplementary Fig. 1). Furthermore, immunohistochemistry (IHC) analysis of kidney tissues showed the presence of nuclear YAP in the majority of epithelial cells forming cysts, whereas YAP localization was mainly cytoplasmic in non-cystic renal cells (Fig. 5b). Quantification using a semi-automated

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**Figure 3.** Both ROCK and MLCK inhibitors revert the effects observed in Pkd1−/− cells. (a) Schematic representation of the complex mechanism of regulation by ROCK inhibitor Y27632 and MLCK inhibitor ML-7 on the phosphorylation status of MLC. (b) Pkd1−/− MEFs show hyper-phosphorylation of MLC, compared to control Pkd1+/+ MEFs. Treatment with both ROCK inhibitor Y27632 100 µM (Top) and MLCK inhibitor ML-7 10 µM (bottom) is able to decrease MLC phosphorylation in Pkd1−/− MEFs to the levels of control cells. Quantification of pMLC/MLC ratio in three independent experiments is presented in Supplementary Fig. 1. Bands were cropped from different parts of different gels. Full-length blots are presented in Supplementary Fig. 2. (c) Quantification of collagen contraction assay at 3 h showing ratio between the gel area and the total well area of MDCK PKD1Zeo cells (left) and Pkd1−/− MEFs (right). Treatment with both ROCK inhibitor Y27632 (100 µM, 4 h) and MLCK inhibitor ML-7 (10 µM, 4 h) is able to revert the cells contractility capacity to respective controls. Values are mean ± SD of three replicates. Statistical analysis: one-way ANOVA; ***p < 0.001, ****p < 0.0001. Experiments were performed at least three times and representative results are shown.
Figure 4. Polycystin-1-dependent myosin regulation correlates with YAP activation. (a) Schematic representation of YAP activation. Left: cells on stiff matrix or at low confluency show active nuclear YAP. Right: cells on soft matrix or at high confluency show inactive cytoplasmic phosphorylated YAP. (b) Left: percentage (%) of nuclear YAP localization quantified by immunofluorescence in MDCK<sup>Zeo</sup> and MDCK<sup>PKD1Zeo</sup> cells plated on collagen coated hydrogels at either stiff (40 KPa) or soft (0.5 KPa) conditions. Right: representative images of YAP localization by immunofluorescence in MDCK<sup>Zeo</sup> and MDCK<sup>PKD1Zeo</sup> cells in stiff condition. Values are mean ± SD of three replicates (in which at least 150 cells were counted). Statistical analysis: ordinary One-way ANOVA; ****p < 0.0001. (c) percentage (%) of nuclear YAP localization quantified by immunofluorescence in MDCK<sup>Zeo</sup> and MDCK<sup>PKD1Zeo</sup> plated at either low (sparse) or high (confluent) confluency conditions. Values are mean ± SD of three replicates (in which at least 150 cells were counted). Statistical analysis: ordinary One-way ANOVA; *p < 0.05. (d) qRT-PCR analysis for YAP targets: Ankrd1 (left) and Cyr61 (right) mRNA normalized towards Hprt1 on MDCK<sup>PKD1Zeo</sup> cells compared to control MDCK<sup>Zeo</sup> cells plated at either low (sparse) or high...
(confluent) confluency conditions. Values are mean + SD of three replicates. Statistical analysis: ordinary One-way ANOVA; *p < 0.05, ***p < 0.001, ****p < 0.0001. (e) Left: percentage (%) of nuclear YAP localization quantified by immunofluorescence in IMCD<sup>shPkd1</sup> cells (clones C12 and C16) compared to control IMCD<sup>sh</sup> cells (clones M3 and M4) plated on collagen coated hydrogels at either stiff (40 KPa) or soft (0.5 KPa) conditions. Right: percentage (%) of nuclear YAP localization quantified by immunofluorescence in IMCD<sup>shPkd1</sup> cells (clones C12 and C16) compared to control IMCD<sup>sh</sup> cells (clones M3 and M4) plated at either low (sparse), high (confluent) or very high (hyperconfl) confluency conditions. Values are mean + SD of three replicates (in which at least 150 cells were counted). Statistical analysis: ordinary One-way ANOVA; *p < 0.05, ***p < 0.001, ****p < 0.0001. (f) percentage (%) of nuclear YAP localization quantified by immunofluorescence in Pkd1<sup>−/−</sup> MEFs compared to control Pkd1<sup>+/+</sup> MEFs plated on collagen coated hydrogels at either stiff (40 KPa) or soft (1.5 KPa and 0.5 KPa) conditions. Values are mean + SD of three replicates (in which at least 150 cells were counted). Statistical analysis: ordinary One-way ANOVA; *p < 0.05, **p < 0.01. All experiments were performed at least three times and representative results are shown.

system confirmed the significant increase in cells with YAP nuclear translocation in mutant kidneys (Fig. 5c) in line with previous studies. In line with this observation, we also found an enhanced expression of YAP target genes in cystic kidneys at different ages, both in a neonatal PKD model and in the adult compared to controls (Fig. 5d). Importantly, treatment of mice with the ROCK inhibitor Fasudil led to a decrease in MLC phosphorylation (Fig. 6a and Supplementary Fig. 1), YAP nuclear translocation (Fig. 6b,c) and expression of YAP target genes Ctgf, Ankrd1, Cyr61 (Fig. 6d). These results demonstrate that in the cystic epithelia the increased YAP nuclear translocation and activity is mainly dependent on the actomyosin contraction machinery, supporting the hypothesis that cystic epithelia fail to sense and activate a proper cellular response to extracellular rigidity. Of note, treatment of mice with the inhibitor Fasudil by intra-peritoneal injection (i.p.) for three consequent days was also sufficient to ameliorate the cystic phenotype. Mice showed a reduced kidney/body weight (Fig. 6e) with a decrease of the cystic index due to the increment of normal tubules (Fig. 6f). Furthermore, analysis of proliferation by Ki67 staining showed a significant decrease in proliferation (Fig. 6g), whereas analysis of apoptosis by TUNEL did not show any difference between untreated and treated cystic kidneys (Fig. 6h). Thus, we conclude that Fasudil achieves improvement of the phenotype by reducing the proliferation rates of the cystic epithelia.

Discussion

In this study, we performed a controlled screening for intracellular interactors of endogenous PC-1 and identified a new role for this receptor as a mechanosensor of the cellular response to extracellular stiffness. Of interest, PC-1 appears to act as a negative, rather than a positive, regulator of the cellular response to rigidity (Fig. 7).

The mass-spectrometry analysis of mouse embryonic fibroblasts carrying tagged endogenous PC-1 revealed that PC-1 interacts with proteins that regulate the actomyosin contraction machinery. Accordingly, we found that overexpression of PC-1 decreases pMLC levels and cellular contraction, while cells knocked-out or silenced for Pkd1 have both effects upregulated. We further found increased pMLC in polycystic kidneys generated by kidney-specific inactivation of Pkd1. The actomyosin pathway plays a central role in the response of cells to extracellular rigidity. To study whether PC-1 could have a role in regulating this process we have used YAP translocation into the nucleus as a read-out of this response. Plating cells on hydrogels that mimic the different stiff-ness of the ECM, we have observed a reduced YAP nuclear translocation in cells overexpressing PC-1 in stiff conditions while Pkd1 mutant cells partially failed to respond to soft condition showing an enhanced nuclear localization of YAP. Furthermore, Pkd1 mutant kidneys display enhanced actomyosin-dependent YAP nuclear translocation and transcriptional activity. It is interesting to note here that to date only positive regulators of the extracellular response to stiffness have been identified, whereas our data seem to indicate that PC-1 might be acting as a receptor for extracellular stiffness able to inhibit the cellular response via inhibition of phosphorylation of myosin. Indeed, our studies are supported by evidence that inactivation of a positive regulator of the pathway, integrin β1, results in a drastic improvement of the cystic phenotype in Pkd1 mutant mice. Our data are also in agreement with the previously reported role of PC-1 in the regulation of cell adhesion and in particular as a positive regulator of focal adhesions turnover. It remains to be established whether PC-1 exerts this novel function via its localization at focal adhesions and cell–cell contacts or, on the contrary, whether this new function of PC-1 is also modulated by the pool of proteins present in primary cilia and future work should focus on understanding this aspect. Irrespective of the subcellular compartment in which PC-1 is active to inhibit this pathway, it is important to note here that our data might potentially explain a very interesting recent finding observed in human-derived iPS cells. Indeed, it was shown that patient derived cells carrying a PKD1 mutation can originate a very aggressive form of cystogenesis when cultured in vitro, but only when the mechanical constraints are lacking, i.e. when cells are grown in suspension. Based on our findings we speculate that this might be due to an inability of the cells to respond to the extracellular mechanical force given the absence of a key mechanoreceptor such as PC-1. Our data are also potentially in agreement with studies carried out in Zebrafish. Indeed, silencing of Pkd1 by morpholinos in the fish resulted in increased collagen matrix deposition and tail curvature. We speculate that in this context as well, absence of PC-1 might result in a failure to sense the correct amount of collagen deposition and matrix rigidity, ultimately leading to a constitutive and excessive deposition of matrix, a phenotype also observed in patient specimens. Given the fact that deletion of integrin β1 in PKD murine models results in phenotype improvement, it would be interesting to test whether morpholinos directed against the same integrin subunit in the fish improves also this specific phenotype. Importantly, the fact that an increased matrix deposition was also observed in patient-derived tissues, points to the potential central importance of the process for the pathophysiology of ADPKD.
Figure 5. Polycystin-1 regulates phosphorylation of myosin light chain in kidneys. (a) Phosphorylation levels of MLC in activatory sites Thr18/Ser19 were tested by Western blot on kidney lysates. Conditional kidney inactivation of Pkd1 gene (Pkd1flox−/−:KspCre) causes a robust hyper-phosphorylation of MLC in mice at both P5 and P12, compared to control Pkd1flox+/+:KspCre mice. Quantification of pMLC/MLC ratio in three independent experiments is presented in Supplementary Fig. 1. Bands were cropped from different parts of different gels. Full-length blots are presented in Supplementary Fig. 2. (b) IHC staining on Pkd1flox−/−:KspCre and control Pkd1flox+/+:KspCre kidney at P8 for YAP. Aperio image analysis software mask for the semi-automated quantification of YAP staining. IHC staining on control Pkd1flox+/+:KspCre kidney for DBA. Scale bar = 50 μm. (c) Percentage (%) of nuclear YAP localization quantified as strong positive by semi-automated Aperio image analysis software on IHC slides of Pkd1flox−/−:KspCre and control Pkd1flox+/+:KspCre kidneys at P8 (n = 3 cystic and n = 3 littermate controls). Values are mean ± SEM of 22 cysts (224 nuclei counted for Pkd1flox−/−:KspCre; 706 nuclei counted for Pkd1flox+/−:KspCre). Statistical analysis: Student’s unpaired one-tailed t-test. ****p < 0.0001.
Finally, it should be considered that, irrespectively of whether or not deregulation of actomyosin contraction is a primary event resulting in cystogenesis or, vice versa, it is deregulated once cysts have formed, it is important to highlight the potential of targeting this pathway as a new therapeutic approach. Indeed, we have found that the in vivo inhibition of the ROCK pathway using the compound Fasudil reduced YAP nuclear translocation and ameliorates PKD progression. These data are in agreement with recent work showing that the inhibitor Y27632 was able to retard disease progression in animal models. While it is difficult to think that Y27632 could be used in the clinic, this provides a very important proof of principle. Our data confirm these findings and in addition show that a compound available on the market for targeting a different medical condition could offer a good opportunity for drug repurposing.

Finally, while our data do not clarify the precise mechanism by which PC-1 regulates the actomyosin contraction, they indicate that both ROCK and MLC kinase inhibitors are able to revert the pMLC levels in mutant cells. This is relevant because MLC is a Ca++ dependent kinase that might be altered primarily or secondarily in ADPKD tissues due to PC-2 dysfunction and this might concur in regulation of pMLC levels and cellular proliferation in the cystic epithelia.

In conclusion, our results indicate that applying a very stringent method for the identification of PC-1 interactors allowed the identification of few molecules pointing to a central role for PC-1 in regulation of the cellular response to the physical nature of the extracellular environment, such as the rigidity of the milieu. Even if this property and the novel central function of PC-1 potentially involved in inducing cystogenesis, it might be difficult to be corrected. However, inhibition of a downstream pathway involved in the cellular response might contribute to retard disease progression and might be explored as a potential new therapy.

Materials and Methods

Cell lines. MDCK cell lines stably transfected either with a full-length human PKD1 cDNA construct (MDCKPKD1Zeo clones C8/68, G3/11) or with an empty vector (MDCKZeo, clones P2, F6) are described in Boletta et al. Murine IMCD cells transduced with viral vectors expressing shRNA encoding the Pkd1-targeting shRNA sequence (shPkd1) (clones C12 and C16) or the scrambled sequences (shScr) (control clones M3 and M4) are already described. All the cell lines were grown in 37°C, 5% CO2 incubators, in high glucose DMEM medium (GIBCO, #41965-062), 10% FBS, 1% Penicillin-Streptomycin (GIBCO, #15070-063); for MDCK, medium was supplied with 0.5 μg/ml Geneticin (GIBCO, #11811-064) and 0.05 μg/ml Zeocin (Invitrogen, #R25001). For IMCD clones, medium was supplemented with 1 μg/ml Puromycin (Invitrogen, #A11138-03).

Mice. The generation of both Pkd1WT/−:KspCre and Pkd1WT/−:TmCre mice in a pure C57BL/6N genetic background has been previously described. Intra-litter Pkd1WT/−:KspCre and Pkd1WT/−:TmCre mice respectively were used as controls. For the Pkd1WT/−:TmCre model the Cre recombinase activity was induced by a single i.p. injection at P12 of Tamoxifen (Sigma-Aldrich, #T5648; 10 mg/40 g) dissolved in corn oil (Sigma-Aldrich, #C8267) by continuous shaking at 37 °C for 8 h. For all animal work, the female to male ratio was 1:1, and mice were randomized for each experiment. All animal care and all protocols used were carried out according to the Institutional regulations and specifically approved by the institutional care and use ethical committee at the San Raffaele Scientific Institute, further approved by the Italian Ministry of Health (IACUC #736).

In vivo treatments. We intra-peritoneally (i.p.) injected the Rho kinase inhibitor Fasudil (LC Laboratories, #F-4660) or vehicle (NaCl) daily from P6 until P8 at 25 mg/kg of body weight. For boost treatment we i.p. injected Fasudil or vehicle (NaCl) at P8 five times every hour at 10 mg/kg of body weight.

PC-1 immunoprecipitation. Pkd1HA/HA and Pkd1WT/WT MEFs were grown in presence of aminoacids labeled with different isotopes (heavy-13C and light-12C, respectively). PC-1 target proteins were immunoprecipitated using anti-HA antibody. Briefly, Pkd1HA/HA and Pkd1WT/WT MEFs were lysate by using lysis Buffer 1% triton X-100 (Sigma-Aldrich, #1052324354). 10 mg of total extract (1 ml final volume) was incubated for 2 h with 50 μl of agarose beads at 4 °C to pre-clear the samples. The clarified supernatants were incubated overnight with anti-HA beads for the IP. Then, samples were washed 5 times with Buffer 1% triton.

Depending on the type of SILAC experiment, the heavy and light proteins immunoprecipitated were mixed 1:1 after the IP, then, for SILAC1, PC-1 IP eluates were separated by 4–12% SDS-PAGE (Life Technologies, #NP03353BOX), stained with Coomasie Brilliant Blue (Sigma-Aldrich, #B-0149) and excised in eight slices for LC-MS/MS analysis. For SILAC2, beads were resuspended in Urea 6M, Tris HCl 10 mM, proteins were digested with Lys C overnight, Urea was then diluted to 2M and trypsin digestion was performed for 3 h.

Mass spectrometry and data analysis. Mass spectrometry analysis was performed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (ThermoScientific, Bremen, Germany). Tryptic digests were cleaned using Stage Tips as described previously and then injected in a capillary chromatographic system (EasyLC, Proxeon Biosystems, Odense, Denmark). Peptides separation occurred on a homemade column obtained with
Figure 6. Treatment with ROCK inhibitor Fasudil ameliorates the cystic phenotype. (a) Phosphorylation of MLC in activatory sites Thr18/Ser19 were tested by Western blot on kidney lysates of Pkd1<sup>fl<sup>ox</sup>-</sup>:KspCre mice at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle. Quantification of pMLC/MLC ratio in three independent experiments is in Supplementary Fig. 1. Bands were cropped from different parts of different gels. Full-length blots are presented in Supplementary Fig. 2. (b) IHC staining for YAP on Pkd1<sup>fl<sup>ox</sup>-</sup>:KspCre kidney at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle. Aperio image analysis software mask for the semi-automated quantification of YAP staining. Scale bar = 50 μm. (c) Percentage (%) of nuclear YAP localization quantified as strong positive by the semi-automated Aperio image analysis software on IHC slides of Pkd1<sup>fl<sup>ox</sup>-</sup>:KspCre kidneys at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle (n = 3 cystic fasudil and n = 3 cystic vehicle treated mice). Values are mean ± SEM of forty five cysts (1715 nuclei counted for vehicle; 2003 nuclei counted for Fasudil). Statistical analysis: Student's unpaired one-tailed t-test. ****p < 0.0001. (d) qRT-PCR analysis for YAP targets: Ctgf, Ankrd1 and Cyr61 mRNA normalized towards Hprt1 on Pkd1<sup>fl<sup>ox</sup>-</sup>:KspCre kidneys at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle (n = 5 cystic fasudil and n = 5 cystic vehicle treated mice). Values are mean ± SEM. Statistical analysis: ordinary One-
way ANOVA; **p < 0.01. ****p < 0.0001 (e) Kidney/body weight of Pkd1flox/−;KspCre mice and Pkd1flox/+;KspCre controls after three day-treatment with ROCK inhibitor Fasudil or vehicle. Values are mean ± SEM of at least 6 animals. Statistical analysis: Student's paired two-tailed t-test; **p < 0.01. (f) Percentage (%) of both normal tubes and cysts on total structures on slides of Pkd1flox/−;KspCre kidneys at P8 after 3-day treatment with ROCK inhibitor Fasudil or vehicle (n = 3). Values are mean ± SD of at least 1000 cortical structures counted per kidney. Statistical analysis: Student's paired one-tailed t-test; *p < 0.05. (g) Percentage (%) of Ki67 staining quantified by semi-automated Aperio image analysis software on IHC slides of Pkd1flox/−;KspCre kidneys at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle (n = 3). Values are mean ± SEM of 30 cysts (1904 nuclei counted for vehicle; 1709 nuclei counted for Fasudil). Statistical analysis: Student's unpaired one-tailed t-test; *p < 0.01. (h) Percentage (%) of apoptosis (TUNEL positive cells) quantified by immunofluorescence in slides of Pkd1flox/−;KspCre kidneys at P8 after boost treatment with ROCK inhibitor Fasudil or vehicle (n = 3 cystic fasudil and n = 3 cystic vehicle treated mice). Values are mean ± SEM of 58 cysts (2580 nuclei counted for vehicle; 2497 nuclei counted for Fasudil). Statistical analysis: Student's unpaired one-tailed t-test; ns, non significant.

a 10-cm fused silica capillary (75 m inner diameter and 360 m outer diameter; Proxeon Biosystems) filled with Reprosil-Pur C18 3-m resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany) using a pressurized ‘packing bomb’. A gradient of eluents A (distilled water with 2% (v/v) acetonitrile, 0.1% (v/v) formic acid) and B (acetonitrile, 2% (v/v) distilled water with 0.1% (v/v) formic acid) was used to achieve separation from 8% B (at 0 min, 0.2 ml/min flow rate) to 50% B (at 80 min for SILAC1 and 180 min for SILAC2, 0.2 ml/min flow rate). The LC system was connected to the orbitrap equipped with a nanoelectrospray ion source (Proxeon Biosystems). Full-scan mass spectra were acquired in the LTQ-Orbitrap mass spectrometer in the mass range m/z 350–1500 Da and with the resolution set to 60000. The ‘lock-mass’ option was used for accurate mass measurements. The 10 most intense doubly and triply charged ions were automatically selected and fragmented in the ion trap. Target ions already selected for the MS/MS were dynamically excluded for 60 s. Protein identification and quantification were achieved using the MaxQuant software version 1.1.1.36 59. Mass spectra were analyzed against a concatenated forward and reversed database version of uniprot_cp_mus_2011_03. The initial mass tolerance in MS mode was set to 7 ppm and MS/MS mass tolerance was 0.5 Da. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein and oxidized methionine were searched as variable modifications. Labeled arginine and lysine were also specified as variable modifications. SILAC peptides and proteins quantification were performed automatically with MaxQuant using default settings as parameters. Peptides and proteins were accepted with a false-discovery rate of 0.01, minimum one unique peptide identified per protein. To identify PC-1 interactors, we analyzed the Gaussian distribution and we used the standard deviation of the sample as a cutoff to identify outliers. Outliers were selected based on p < 0.05 as the most stringent criteria and a cutoff ratio H/L > mean + 2 SD considering the Gaussian distribution.

**Antibodies and inhibitors.** For Western blot analysis we used the following antibodies: PC-1 LRR (Santa Cruz Biotechnology, #sc-130554; 1:1000), HA High Affinity (Roche, #11867423001; 1:1000) p-MLC(Thr18/Ser19) (Cell Signaling, #3674S; 1:1000), MLC (Cell Signaling, #3672; 1:1000). HRP-conjugated secondary antibodies were from GE Healthcare, anti-rabbit IgG HRP linked (#3943V) anti-mouse IgG HRP linked (#NA9310V) and anti-rat IgG HRP linked (#NA935V). For IF and IHC staining we used YAP (Santa Cruz Biotechnology, #sc-101199; 1:200 or Cell Signaling, #4912; 1:50 respectively), Ki67 (Thermo Fisher Scientific, #RM-9106; 1:100) antibodies and DAPI (Santa Cruz Biotechnology, #sc-3598; 1:5000). Alexa-488 secondary anti mouse antibody were from Invitrogen (#A11001). For IP we used anti-HA affinity matrix (Roche, #11815016001). Rho kinase inhibitor Y27632 and MLCK inhibitor ML-7 were from Tocris (#1254 and #4310, respectively).

**Immunohistochemistry.** For histological analysis mice were sacrificed at the indicated time, kidneys were collected, weighted and fixed in formalin 10% (BioOptica, #05-01005Q), included in paraffin and cut 5 μm/slices. Kidney sections were air-dried and rehydrated in PBS (Sigma-Aldrich, #P4417). For YAP staining kidney sections were incubated with YAP antibody and 5 min in Eosin G (BioOptica, #05-10002/L); For Ki67 staining kidney sections were incubated with Ki67 antibody and 5 min in Hematoxylin (BioOptica, #05-01005Q), included in paraffin and cut 5 μm/slices. Sections were then washed, processed through a dehydration alcohol scale and mounted in DPX (Sigma-Aldrich, #06522). For Ki67 staining kidney sections were air-dried and rehydrated in PBS (Sigma-Aldrich, #A11001). For IP we used anti-HA affinity matrix (Roche, #11815016001). Rho kinase inhibitor Y27632 and MLCK inhibitor ML-7 were from Tocris (#1254 and #4310, respectively).

**Cystic index.** To quantify the Cystic Index we manually counted at least 1000 tubular structures in cortical sections of kidneys stained with Hematoxylin-Eosin. To quantify the rate of cystic kidneys we applied a grid of squares 13.625 μm large to sections of kidneys stained with Hematoxylin-Eosin. We marked each cross with a dot and we manually counted the number of dots inside the lumen on three litters. We determined the degree of dilatation according to 1 dot: normal tubules; >2 dots: dilated tubules and cysts as in 40.

**Immunofluorescence.** For immunofluorescence staining, cells seeded on a glass or on a hydrogels-covered glass were washed in PBS, fixed 10 min in 4% Paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS. After 1 h blocking in 3% BSA (Sigma-Aldrich, #A7906) in PBS at RT, cells were incubated over night at 4 °C with primary antibody diluted in 3% BSA in PBS. Cells were then incubated with secondary antibody diluted in 3% BSA in PBS for 1 h at RT and nuclei were stained with DAPI. Glasses were then mounted with Fluorescence Mounting medium (Dako, #S3023). For the apoptotic assay, kidney sections were analyzed by the DeadEnd.
Flurometric transferase-mediated dUTP nick-end labeling (TUNEL) system kit (Promega, #G3250). Images were obtained using Zeiss Axio Observer.Z1 microscope. Quantification of both YAP and TUNEL nuclear staining was performed manually.

**Real-time PCR analysis.** Total RNA was isolated from cells or kidneys using the RNAspin Mini kit (GE Healthcare, #25-0500-72). cDNA was obtained by reverse transcription of extracted RNA using Oligo(dt)15 primers (Promega, #C1101) and ImProm-II Reverse Transcriptase (Promega, #A3802). Quantitative Real Time PCR analysis was performed on technical duplicates using SYBR Green I master mix (Roche, #04887352001) on LightCycler 480 Instrument (Roche). Primer sequences for qRT-PCR:

- **mHprt1**
  - fw: 5'-TTATGTCCCCCGTTGACTGA-3’
  - rev: 3’-ACATTGTGGCCCTCTGTGTG-5’

- **mCtgf**
  - fw: 5’-GCTTGGCGATTTTAGGTGTC-3’
  - rev: 3’-CAGACTGGAGAAGCAGAGCC-5’

- **mAnkrd1**
  - fw: 5’-CAGACTGGAGAAGCAGAGCC-3’
  - rev: 3’-GCTTGGCGATTTTAGGTGTC-5’

- **mCyr61**
  - fw: 5’-CAGACTGGAGAAGCAGAGCC-3’
  - rev: 3’-GCTTGGCGATTTTAGGTGTC-5’

- **Canine Hprt1**
  - fw: 5’-ACACTGGGAAAACAATGCAGAC-3’
  - rev: 3’-TCAGGTTTTATAGCCAACACTCAGTC-5’

- **Canine Ankd1**
  - fw: 5’-TGAGGCCAGAGGTGGTCACGC-3’
  - rev: 3’-CAGACTGGAGAAGCAGAGCC-5’

- **Canine Cyr61**
  - fw: 5’-TCTGTTGACGAGGATGATGC-3’
  - rev: 3’-TGTCAAGTGGGAGGTTCACGC-5’

**Western blot analysis.** For Western blot analysis cells or kidneys were lysed in lysis buffer solution of 150 mM NaCl (Sigma-Aldrich, #S9625), 20 mM NaHPO4 (BDH, #10494L)/NaH2PO4 (BDH, #102455S), 10% Glycerol (Sigma-Aldrich, #G7757), 1% Triton X-100 (pH 7.2), complete protease inhibitor cocktail (Roche, #1183614501) and phosphatase inhibitors [1 mM final concentration of glycerophosphate (Sigma-Aldrich, #G9891), sodium orthovanadate (Sigma-Aldrich, #S6508) and sodium fluoride (Sigma-Aldrich, #S6521)].
Hydrogels of polyacrylamide. In order to test the cellular response to the mechanical properties of the extra-cellular matrix we have prepared hydrogels of polyacrylamide with modulable rigidity. The protocol described by Tse et al.61 has been adapted as follows: 15 mm of diameter round cover glasses were incubated with 250 μl 0.1M NaOH (Riedel de Haen, #30620) and placed at 80 °C until the liquid has evaporated. Glasses were then incubated with 125 μl of 3-aminopropyltriethoxysilane (APES) (Thercoscience, #80370) for 5 min and then placed into a multi-well plate and washed with ddH2O three times for 5 min. Glasses were then incubated with 250 μl of 0.5% glutaraldehyde (Sigma-Aldrich, #G6257) in PBS for 30 min. After removing the glutaraldehyde and allow glasses to dry, we treated the glass surface of slide glasses with 100 μl of Diclorodimethylsilane (DCDMS) (Merck, #8034520250) for 5 min and then washed with ddH2O. We prepared acrylamide/bis-acrylamide (Sigma-Aldrich, #A2792) solutions at different proportions according to the desired rigidity and added 10% APS (1:100) and TEMED (1:1000) (BDH, #4430836) quickly before pipetting 10 μl of solution onto the slide glass. We put the cover glasses with the side treated towards the drop and let the gel polymerized for 10 min. We then washed the obtained hydrogels with ddH2O and store them at 4 °C in PBS. The day before plating cells, we treated the hydrogels with 500 μl of sufo-SANPAH (0.2 mg/ml sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino-hexanoate) (Thermo Fisher Scientific, #22589) under UV (365 nm) for 10 min. We washed three times with HEPES (Sigma-Aldrich #H3375) 50 mM pH 8.5 and coated the hydrogels with 50 μl of Collagen type 1 Rat tail solution (Corning, #354236) in HEPES 0.10 mg/ml at 37 °C O/N. The following day, we transferred the hydrogels into a 12-well plates and washed them three times with PBS. After treating the hydrogels for 30 min with UV, we seeded 35000 cells/hydrogels.

Contraction assay. To monitor cellular contractility in vitro, cells were plated on thin collagen gels. Cellular contraction caused a decrease in the collagen area that can be quantified to estimate contractility during time.62 The day before plating the cells we coated a 12-well plate with 500μl/well of a Collagen solution in PBS at 37 °C O/N: the solution consist in 7 Volumes of Collagen Type 1 (1 mg/ml), 2 Volumes of DMEM (GIBCO, #52100-039) and 1 Volume of Neutralizing Buffer (2.2 gr NaHCO3 (Carlo Erba Reagents, #478537), 4.77 gr NaOH 0.05M in a final volume of 100 ml), FBS 5%. The day after, we seeded 150000 cells/well and waited the cells to adhere for about 4 h. We then detached the edges of the gel form the well and monitor the changes in the gel area taking pictures over time. The analysis of the images of the gel area/well area were performed with the ImageJ software.

Statistical analysis. Differences between averages were established with Student’s t-test or one way ANOVA analysis of variance as indicated in the figure legends; Bonferroni’s post-test was carried out for multiple comparisons.

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Author contributions
E.A.N., designed and performed most studies, analyzed data, prepared the figures including drawings and wrote the manuscript; G.D., performed the labeling and IP from cells, conceived the initial idea of the study and performed Western blot experiments; M.Chiaravalli, performed animal crossings and in vivo experiments and analyzed histological data; V.M., designed, performed and interpreted the Mass Spectrometry experiments; M.Castelli, set up the collagen contraction assay experiments; A.P.G, set up the hydrogels experiments; A.Bachi, supervised the Mass Spectrometry analysis and wrote the manuscript; A.Boletta, supervised the work and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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