Polycystin-1 binds Par3/aPKC and controls convergent extension during renal tubular morphogenesis

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Several organs, including the lungs and kidneys, are formed by epithelial tubes whose proper morphogenesis ensures correct function. This is best exemplified by the kidney, where defective establishment or maintenance of tubular diameter results in polycystic kidney disease, a common genetic disorder. Most polycystic kidney disease cases result from loss-of-function mutations in the PKD1 gene, encoding Polycystin-1, a large receptor of unknown function. Here we demonstrate that PC-1 has an essential role in the establishment of correct tubular diameter during nephron development. Polycystin-1 associates with Par3 favouring the assembly of a pro-polarizing Par3/aPKC complex and it regulates a programme of cell polarity important for oriented cell migration and for a convergent extension-like process during tubular morphogenesis. Par3 inactivation in the developing kidney results in defective convergent extension and tubular morphogenesis, and in renal cyst formation. Our data define Polycystin-1 as central to cell polarization and to epithelial tube morphogenesis and homeostasis.
The kidney develops as a result of interactions between the ureteric bud (UB) and the metanephric mesenchyme (MM)\(^1\). The UB invades the MM and undergoes a series of branchings forming the collecting duct system and ureter of the mature kidney\(^1\). The UB also induces a condensation and epithelialization of the MM to form comma and S-shaped bodies. The mechanisms responsible for UB branching and MM condensation have been thoroughly studied over the years with important progress being made\(^1\). By contrast, the mechanisms responsible for the subsequent steps of maturation have been the focus of more recent studies, and many outstanding questions remain\(^1\). Comma and S-shaped bodies are composed of an immature epithelium with a central lumen, which will undergo a programme of patterning. Both MM- and UB-derived structures undergo a programme of tubular elongation necessary to generate the mature nephron and collecting duct system, respectively\(^2\).

Recent important progress has been made in understanding the mechanisms underlying the elongation process, as well as the establishment and maintenance of tubular diameter\(^1\). This process is achieved by at least two mechanisms. During embryonic development, both the collecting duct and the proximal tubules decrease in diameter. Although cell division is limited, the establishment and maintenance of tubular diameter 1–3. Defective regulation of tubular diameter is associated with autosomal dominant polycystic kidney disease (PKD), one of the most common inherited disorders\(^6\). The hallmark of this disease is bilateral renal cyst formation, due to loss-of-function mutations in two genes: PKD1 in 85% of cases or PKD2 in the remaining 15%. Expression of the PKD1 gene is developmentally regulated in the kidneys\(^7\), suggesting its possible involvement in normal tubular morphogenesis. PKD1 encodes for Polycystin-1 (PC-1), a large plasma membrane receptor of unknown function, with a very large extracellular amino terminus containing protein–protein interaction domains, 11 transmembrane domains and a short intracellular carboxy terminus\(^7\). PC-1 has been implicated in a number of biological processes\(^7\), but to date its precise function remains to be defined. The role of the Pkd1 gene in

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**Figure 1 | Defective tubular narrowing and cellular morphology in Pkd1 mutants.** (a) Histogram of the percentage (y axis) of DBA-positive tubules with a given number of cells (x axis) per tubule cross-section at E13.5, E15.5 and E16.5 in wt (top, grey bars) or Pkd1\(^{SC/AC}\) (bottom, orange bars) kidneys. The squares and bars on top show the average and the distribution (maximum and minimum numbers), respectively. (b) Graph extrapolated from the data in a. Histograms and bars represent the average and s.e.m. At E13.5, n = 183 (wt) and n = 182 (KO), and at E15.5 and E16.5, n = 200 for both the genotypes, where n indicates the number of tubules counted. Statistical analysis was carried out by analysis of variance followed by the Bonferroni parameter. NS, nonsignificant; **P<0.01; ***P<0.001. (c) Representative sections of DBA-positive (red) developing tubules at E16.5 of Pkd1\(^{+/+}\) or Pkd1\(^{SC/AC}\) kidneys. Each cell is labelled with an asterisk and the number of cells per cross-section indicated in the lumen. Scale bar, 20 μm. (d) Confocal images (left panels) and cell outlines (right panels) of frontal sections of E15.5 wt (Pkd1\(^{+/+}\)) or Pkd1 mutant (Pkd1\(^{SC/AC}\)) kidneys stained with an antibody to E-cadherin in DBA-positive sections. Images in the left panels represent sections two frames basal to the aPKC staining, as previously described\(^4\). Medially elongated cells are labelled in white, proximal-distally in black, non-elongated cells in grey. Scale bar, 10 μm. (e) Quantification of the angle of cellular elongation relative to the proximal-distal axis of the tubule for Pkd1\(^{+/+}\) (left) and Pkd1\(^{SC/AC}\) (right). The percentage of cells in each 10° increment is shown. White bars represent cells elongated mediolaterally within a 40°–90° angle. Cells for wt (n = 530) and for mutants (n = 575) were counted in a minimum of six kidneys from three litters. P < 0.0001. Statistical analysis was performed using the Mann–Whitney test.
mediating OCD in the tubules of newborn kidneys has been recently investigated and led to controversial results, whereas the role of PC-1 in tubular morphogenesis and CE during development was not investigated.

Here we show that PC-1 is essential for a proper establishment of tubular diameter and mediolateral cell orientation during embryonic renal development in the mouse. PC-1 directly associates with Par3 and favours the association of a Par3/atypical protein kinase C (aPKC) complex in a process likely to be important for oriented cell migration and for renal tubular morphogenesis in the developing kidney. In line with this, we also find that inactivation of the Par3 gene in the developing kidneys leads to defective CE and to a non-fully penetrant renal cystic phenotype.

**Results**

**Defective CE in Pkd1\(^{AC/AC}\) kidneys.** To test whether PC-1 is involved in CE during renal development, we analysed a Pkd1 mutant allele previously described by our group, which lacks the last two exons of the gene, resulting in a mutant protein lacking the intracellular C-tail (Pkd1\(^{AC/AC}\))\(^{10}\). These mice die between E16.5 and E17.5, and display renal cyst formation starting at E15.5 similar to other PKD mice models. The number of cells per cross-section of DBA (Dolichos biflorus agglutinin)-positive renal tubules in wild-type (wt) kidneys decreased over time from E13.5 to E16.5 (Fig. 1a,b), indicating that their diameter decreases (Supplementary Fig. S1). Analysis of Pkd1\(^{AC/AC}\) mutants showed no significant difference from wt at E13.5 (Fig. 1a,b), but a significant defect in tubular narrowing as compared with the wt

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**Figure 2** | **PC-1 controls polarized migration.** (a) Tracking of cells of Pkd1\(^{+/+}\) and Pkd1\(^{-/-}\) MEFs in 12-h wound-healing time-lapse experiments. Each coloured line is a cell. (b) Scheme and quantification of the experiments in a. The angle of deviation \(\alpha\) of each trajectory from a theoretical linear migration (equal to angle 0°) was calculated. Cells (n = 102) from nine movies in three independent experiments per cell line. Statistical analysis was performed using the Mann–Whitney test. P < 0.0001. (c) Left, Pkd1\(^{+/+}\) or Pkd1\(^{-/-}\) MEFs subject to wound healing followed by IF with an anti-\(\alpha\)-tubulin (green), anti-pericentrin (red) and DAPI (blue). Scale bar, 20 \(\mu\)m. Right, quantification of front–rear polarity on three independent MDCKPKD1Zeo clones (36, 68 and G3) and two independent MDCKZeo (F2 and F6), on Pkd1\(^{+/+}\) or Pkd1\(^{-/-}\) MEFs or Pkd1\(^{flox/flox}\) MEFs treated or not with a Cre-recombinase. The red line shows a theoretical random distribution (33%). Statistical analysis was performed using the Student’s t-test (for MEFs, left) or analysis of variance (ANOVA; for PKD clones, right) followed by the Bonferroni parameter. **P < 0.01; ***P < 0.001. Data are means ± s.d. and are representative of a minimum of three independent experiments performed in triplicate, in which at least 300 cells were counted. (d) Diagram of PC-1 different isoforms and/or mutant constructs. (e) Immunoblotting of PC-1 in fibroblasts or in MDCKPKD1Zeo (36) and MDCKZeo (F2) using an anti-LRR antibody (7e12, see Methods), detects both the full-length (FL-PC1, ~205 kDa) and the cleaved, N-terminal fragment (NTF, ~200 kDa). No signal is observed in Pkd1\(^{-/-}\) cells. (f) Immunoblotting of endogenous tagged PC-1 using an anti-HA detects two bands corresponding to the full-length uncleaved PC-1 (FL-PC1, ~205 kDa) and the C-terminal fragment (CTF, ~150 kDa). Treatment with a Cre-recombinase causes disappearance of FL-PC1-HA detection. (g) MDCK type-II cells were transiently transfected with green fluorescent protein alone or along with full-length PC-1 (FL-PC1), the R4227X or the CTF HA-tagged mutants. After sorting, cells were treated as in c. Data are means ± s.d.; Statistical analysis was performed using ANOVA followed by Bonferroni parameter. ***P < 0.001, NS, nonsignificant.
tubules at later stages (E15.5 and E16.5, Fig. 1a,b,c and Supplementary Fig. S1). Staining with Ki67, terminal transferase dUTP nick-end labelling (TUNEL) and cleaved caspase-3 revealed that neither differences in proliferation nor in apoptosis could account for the differences between Pkd1<sup>+/−</sup> and Pkd1<sup>+/−</sup> tubules (Supplementary Fig. S1). Conversely, analysis of the morphology of epithelial cells in DBA-positive tubules revealed that the mediolateral orientation normally observed in wt was lost in Pkd1<sup>+/−</sup> developing kidneys at E15.5 (Fig. 1d,e and Supplementary Fig. S1).

PC-1 regulates the front–rear polarity and oriented migration. We next aimed at investigating the molecular mechanism underlying this defect. Unfortunately, an appropriate functional assay to study CE or planar polarity in vitro is not available. However, the process of CE is intimately linked to the capability of cells to migrate and orient correctly. Therefore, we used cells derived from Pkd1<sup>+/−</sup> or Pkd1<sup>−/−</sup> mice (mouse embryonic fibroblasts, MEFs), in which we have previously reported a defect in migration rates<sup>11</sup>. During our assays, we noticed that in wound-healing assays Pkd1<sup>−/−</sup> MEFs took a contorted path to fill the wound, whereas wt cells migrated in a more linear manner (Fig. 2a,b). Furthermore, Pkd1<sup>−/−</sup> cells failed to relocate their microtubule organizing centre and Golgi in front of their nuclei to generate front–rear polarity as observed in wt cells<sup>12,13</sup> (Fig. 2c).

Next, we isolated MEFs derived from a mouse model expressing a floxable haemagglutinin (HA)-tagged endogenous PC-1 (Pkd1<sup>HA/HA</sup> or Pkd1<sup>floxed/floxed</sup> interchangeably)<sup>10</sup>. As previously described, HA tags were inserted in-frame into the last exon of the Pkd1 gene, resulting in the expression of HA-tagged endogenous PC-1 (ref. 10, Fig. 2f). These lines also carry loxP sites flanking exon 45 and exon 46 (ref. 10). Therefore, on treatment with a Cre-recombinase, Pkd1<sup>HA/HA</sup> generate the Pkd1<sup>+/−</sup> alleles described above, which have lost the protein’s C terminus and its detectability (Fig. 2c,f). Importantly, we found that these cells have lost their capability to polarize in wound-healing assays (Fig. 2c).

Furthermore, a set of Madin–Darby canine kidney, type-II (MDCK type-II) cells overexpressing PC-1 (ref. 14) acquired a polarized migratory phenotype as compared with controls (Fig. 2c). Finally, transient overexpression of wt FL-PC-1, but...
not of the patient-derived mutant R4227X (ref. 15) or the C-terminal fragment16, induced front–rear polarity (Fig. 2g,h). Thus, in addition to the previously described capability of PC-1 to regulate cell motility11, here we show that PC-1 also regulates the front–rear cell polarity during migration and both the C- and the N-terminal domains are required for this activity.

**PC-1 binds the Par3/aPKC complex.** Although the capability of cells to reach appropriate front–rear polarity cannot be considered as an in-vitro assay for CE, the two processes share some molecular factors and mechanisms. One of these is the complex composed of aPKC and the polarity proteins Par3 and Par6, which is very important both for front–rear polarity in mammalian cells and for CE in flies and lower vertebrates13,17–20. Therefore, we investigated whether PC-1 could interact or regulate components of the Par3/aPKC complex. To this end, we used MEFs isolated from Pkd1HA/HA knock-in mice (Fig. 2f)10. Immunoprecipitation (IP) studies revealed that PC-1 coprecipitates with aPKC, a fraction of which reveal phosphorylation in Thr410 (Fig. 3a). In line with this, a kinase-dead mutant of PC-1 abrogates the front–rear polarity in MDCK Pkd1K126R cells, suggesting that this kinase is important (Fig. 3b), and, in line with published studies, the same mutant abrogated the front–rear polarity in wt MEFs (Supplementary Fig. S2). Notably, endogenous PC-1 coprecipitated Par3 but not Par6 in Pkd1HA/HA MEFs (Fig. 3a), and in reverse IP studies Par3 coprecipitated PC-1 in Pkd1HA/HA MEFs (Supplementary Fig. S2). To define the domains involved in the interaction, we overexpressed the full-length HA-tagged PC-1 with the 180 kDa or the 100 kDa form of Par3, lacking the aPKC-binding domain (Fig. 3c), and found that the overexpressed PC-1 coprecipitated both isoforms (Fig. 3d). Furthermore, when the same two constructs were transfected into Pkd1HA/HA MEF lines, we found that

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**Figure 4 | PC-1 can influence the ratio of Par3 versus Par6 bound to aPKC.** (a) MEF cells were subject to wound, fixed and stained for anti-tubulin (green) and anti-Par3 (red). Scale bars, 20 μm. (b) IP from Pkd1+/+ or Pkd1−/− MEFs using an anti-PKCδ antibody followed by immunoblotting with an anti-Par3 antibody in MEFs subject to wound-healing assays as in Fig. 2c. Data are means ± s.e.m. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni parameter. *P<0.01. (d) Green fluorescent protein (GFP)-positive cells, transfected with GFP, Par6 (GFP, bicistronic vector) or Par3 (180 kDa, YFP-tagged, n = 50–120), as well as transfected with three Par3 short interfering RNA (number #1–3, n = 600), were analysed for the capability to polarize in wound-healing assays as in Fig. 2c. Data are means ± s.d., and representative of three independent experiments performed in triplicate. Statistical analysis was performed using ANOVA followed by Bonferroni parameter. *P<0.05, NS, non-statistically significant. (e) Pkd1+/+ or Pkd1HA/HA MEFs were transfected with increasing amounts of Par6A-expressing constructs, followed by IP with anti-HA beads, followed by western blot analysis with anti-aPKC (78 kDa), anti-Par3 (180 kDa), anti-Par6 (43 kDa) or anti-HA antibodies (full-length PC-1 ~ 520 kDa and C-terminal fragment (CTF) ~ 150 kDa). (f) The experiment was performed as in e, except that the Pkd1HA/HA MEFs were transfected with Par6 or Par3K59A construct (43 kDa), mutated in the aPKC-binding site22.
endogenous PC-1 can also interact with both isoforms (Supplementary Fig. S2). Next, we expressed in bacteria the three PDZ domains of Par3, each fused to glutathione S-transferase (GST), and used them in GST pull-down assays along with a histidine-tagged intracellular C-terminal tail of PC-1 (HIS-PC-1-CT)\(^2\). We found that HIS-PC-1-CT binds to the first two PDZ domains of Par3, but not to the third one (Fig. 3e). We conclude that PC-1 C-tail associates directly with Par3 through its first two PDZ domains. These results along with the capability of PC-1 to interact with Par3 lacking the aPKC-binding domain suggest that probably Par3 mediates the interaction between PC-1 and aPKC.

**PC-1 regulates a balance between Par3/aPKC and Par6/aPKC.** Next, we looked at the distribution of Par3 in Pkd\(^{1/-}\) MEFs subject to wound healing and found that these cells fail to properly localize Par3 in distinct patches aligned along the cell–cell contacts, as in wt cells and as previously reported\(^{13}\) (Fig. 4a). Furthermore, IP of aPKC from Pkd\(^{1/-}\) and Pkd\(^{-/-}\) cells revealed that its association with Par3 is reduced, whereas its association with Par6 is enhanced (Fig. 4b,c). We hypothesized that changes in the ratio of the Par3 or Par6 bound to aPKC might influence polarization. Indeed, overexpression of Par6A was able to displace Par3 from aPKC (Supplementary Fig. S3) and caused an impairment of the front–rear polarity (Fig. 4d). Overexpression of Par3 did not have an effect on polarity (Fig. 4d), whereas its silencing impaired the front–rear polarity (Fig. 4d and Supplementary Fig. S3). Importantly, over-expression of wt Par6A in Pkd\(^{1/-}\) fibroblasts competes away the association between PC-1 and aPKC in a dose-dependent manner (Fig. 4e). Using a Par6 mutant (Par6\(^{R19A}\)), which lacks the capability to bind aPKC\(^{22}\), we found that the interaction between Par6 and aPKC is required for its competing activity (Fig. 4f), suggesting that Par6 subtracts aPKC from PC-1/Par3 by binding directly to aPKC. Thus, PC-1 associates with a Par3/aPKC complex (which is in competition with a Par6/aPKC complex) to regulate the front–rear polarity.

**Figure 5 | PC-1 interacts with and regulates Par3/aPKC in the developing kidneys.** (a) Images of DBA-positive tubules at the stages indicated show narrowing of the tubules. Scale bar, 20 μm. (b) Histogram quantifying the process illustrated in a; graphs are average ± s.e.m. of the number of cells per tubule cross-section. (c) Western blot analysis on lysates derived from pools of E13.5, E15.5, E17.5 and P1 developing kidneys from Pkd\(^{1/+}\) mice, using anti-HA (top panel), anti-Par3 (middle panel) and anti-phosphorylated (T410), or total levels of aPKC antibodies (bottom panel). Representative western blotting of three independent experiments. Arrow, full-length PC-1 (~520 kDa); *, C-terminal fragment (CTF) cleavage product of PC-1 (~150 kDa); **, P100 cleavage product of PC-1 (~100 kDa); §, 180 kDa Par; #, 150 kDa Par3; °, 100 kDa Par3. (d) E15.5 total embryo lysates from Pkd\(^{HA/HA}\) (HA) or Pkd\(^{H/-}\) (wt) mice were used to immunoprecipitate PC-1 using anti-HA antibodies, followed by immunoblotting with anti-HA antibodies (top), anti-Par3 (middle) and anti-aPKC antibodies (bottom). Arrow, full-length PC-1 (~520 kDa); *, CTF cleavage product of PC-1 (~150 kDa); §, 180 kDa Par; #, 150 kDa Par3; °, 100 kDa Par3. (e) PC-1 was immunoprecipitated from kidneys of Pkd\(^{HA/HA}\) or Pkd\(^{H/-}\) at E17.5 using anti-HA antibodies, followed by immunoblotting with anti-HA, anti-Par3 and anti-aPKC antibodies. Arrow, full-length PC-1 (~520 kDa); *, CTF cleavage product of PC-1 (~150 kDa); §, 180 kDa Par. (f) IP analysis from E16.5 Pkd\(^{1/-}\) or Pkd\(^{HC/HC}\) kidneys using an anti-PKC\(_y\) antibody followed by immunoblotting with an anti-Par3 or anti-Par6 (43 kDa) antibody. CT, cell lysate from Pkd\(^{1/-}\) embryonic kidneys was incubated in the absence of primary anti-PKC antibody. The blot is representative of three independent experiments. (g) Quantification as means ± s.e.m. of the intensity of bands from three independent experiments performed as in f. Statistical analysis was performed using analysis of variance followed by Bonferroni parameter. **P<0.01.
Par3, and specifically the 180 kDa isoform, overlaps with that of full-length PC-1. Second, IP of endogenous HA-PC-1 resulted in coprecipitation of Par3 and aPKC in total embryos at E15.5 (Fig. 5d), in the developing kidneys at E17.5 (Fig. 5e and Supplementary Fig. S2) and in P1 newborn kidneys (Supplementary Fig. S2). Finally, we analysed the aPKC complexes and found that IP of aPKC reveals that it preferentially associates with Par3 in Pkd1+/–/– E17.5 kidneys, whereas it preferentially associates with Par6 in E17.5 Pkd1+/+/+ homozygous mutant kidneys (Fig. 5f,g). These data are in line with the composition of the complexes observed in cells (Fig. 4b,c). As the Pkd1+/+/+ homozygous mutants lack the intracellular C-tail of PC-1, our studies support the notion that this region is essential for PC-1 proper function during renal tubular morphogenesis.

Defective CE in Par3-mutant kidneys. We next reasoned that if direct interaction between PC-1 and Par3 is essential to mediate correct CE and renal tubular narrowing, we would predict that mice mutant for Par3 display a similar defect than Pkd1 mutants. Homozygous deletion of Par3 results in embryonic lethality before E12.5, preventing the analysis of renal development. Thus, we intercrossed a line harbouring a conditional allele for Par3 (ref. 27) with a line carrying a Hoxb7-Cre (confining Cre expression in the UB)23. Staining of anti-Par3 antibodies confirmed that the protein is lost in a high percentage of cells at E15.5 and at P1 (Supplementary Fig. S4). The resulting mice (Hoxb7Cre; Par3flox/flox−) displayed a defective number of cells per tubular cross-sections in DBA-positive tubules at E15.5 (Fig. 6a,b). Importantly, the Par3 mutants displayed a defective mediolateral orientation of epithelial cells (Fig. 6c). A defective number of cells per tubular cross-sections was also observed at P1 (Fig. 6a,b), when a few cysts and tubular dilatations were also observed in four out of ten Hoxb7Cre;Par3flox/flox− mice (Fig. 6d). Importantly, in E15.5 DBA-positive tubules, the rates of cell division were not affected in the Par3 mutants, whereas apoptosis was slightly increased (Supplementary Fig. S4). Furthermore, analysis of markers of apico-basal polarity and tight junctions formation revealed no major defects at this level in the epithelia lacking Par3 (Fig. 6e and Supplementary Fig. S4), in line with previously published studies. We conclude that indeed Par3 is essential for proper CE during renal development in the mouse and that this process is impaired as early as E15.5.
process is independent of Par3 function in apico-basal polarity, but possibly relies on PCP, as recently demonstrated.3

Discussion
In this study, we have uncovered a critical role for PC-1 in the recently described CE process essential to achieve proper tubular elongation and morphogenesis. In wt developing kidneys, DBA-positive tubules are composed of an average of 13 cells at E13.5 and an average of 7 cells by E16.5. We have shown that in Pkd1 mutant kidneys, DBA-positive tubules fail to undergo this programme of tubular narrowing. Previous studies have shown that the epithelium of the developing nephron divides in a non-oriented manner, but it undergoes mediolateral cellular orientation.4 As this is a hallmark of tissues undergoing a programme of CE to achieve elongation and narrowing29,30, this prompted investigators to hypothesize and, subsequently, to demonstrate that the developing nephron is shaped through CE-like movements.3,4 Indeed, cellular intercalation appears to be the mechanism that best explains how epithelial tubules can narrow over time. Here, we have shown that the epithelium of DBA-positive tubules lacking functional PC-1 fails to orient mediolaterally, suggesting that PC-1 has an essential role in this process. Our study further uncovers a role for PC-1 in regulation of a programme of cell polarity, enabling cells to properly achieve cellular elongation and front–rear asymmetry during migration in wound-healing assays. We have shown that PC-1 interacts with the aPKC/Par3 (180 kDa) complex and probably regulates it to achieve polarized cell migration.

It is interesting to note that PC-1 interacts with both Par3 and aPKC, but not with Par6. Furthermore, the biochemical composition of the aPKC-containing complexes appears to be altered in Pkd1 mutant cells and kidneys. In wt cells, which are able to properly polarize during migration, aPKC preferentially binds to Par3. In contrast, in cells lacking functional PC-1 aPKC appears to preferentially bind to Par6. In addition, overexpression of Par6 is able to compete with Par3 for binding to aPKC and, in line with this, it is able to subtract aPKC from the PC-1/Par3/aPKC complex. This prompted us to hypothesize that Par6 and Par3 might actually compete for binding aPKC. Our data are in line with previous models proposed mostly based on studies in Drosophila, demonstrating that Bazooka (Par3) and Par6 can associate into distinct complexes with different functional roles.3,4 Furthermore, a recent study has demonstrated that a competitive binding between Crumbs and Par6 can modulate the exclusion of Par3 from the Par6/aPKC complex, providing further support for a fine-tuned regulation of the so-called ‘Par6/Par3/aPKC complex’ by competitive binding.31

It is noteworthy that Par3 (180 kDa isoform) and PC-1 appear to follow a similar developmental regulation that parallels the timing of tubular morphogenesis described above. The two proteins are associated in vivo in total embryos and in the developing kidney. We hypothesized that this association might be essential for regulating mediolateral cell orientation and CE during renal tubular morphogenesis. The essential role of the Pars/aPKC complex in regulation of CE downstream of the PCP has been demonstrated in Drosophila melanogaster20,24, whereas a single study has implicated their role in lower vertebrates23. Here we have demonstrated that inactivation of Par3 in the UB of the developing kidney results in defective narrowing of the tubular diameter and defective mediolateral orientation of the epithelia composing these tubules. Thus, our data imply, for the first time, a role of Par3 in CE and tubular morphogenesis in a mammalian system.

Although our studies provide important novel insight in the function of PC-1 and its role in renal development, one important question that remains unanswered is whether and how defective CE might result in cyst formation. We show that only ~40% of the Hoxb7Cre;Par3LoxP/− mice develop cysts. Thus, defective CE alone might not be sufficient to cause cyst formation and compensatory mechanisms might exist. Similarly, in a previous study, Nishio et al.3 have shown that defective OCD does not initiate cyst formation in the newborn kidney, as mice mutant for Pkd1 display defective OCD but not cyst formation. It was proposed that a compensatory mechanism of intercalation might prevent cyst formation in these mice. Thus, one possibility is that neither defects in OCD nor in CE alone are sufficient to initiate cyst formation, but they both might contribute.

In summary, in this study we have described a novel role for PC-1 in regulation of a programme of cell polarity, which is important to achieve proper tubular morphogenesis during renal development. Further studies will be required to assess whether this biological function of PC-1 is important for preventing cyst formation and it is relevant for the disease.

Methods

Antibodies and inhibitors. Anti-α-tubulin and anti-flag antibodies were obtained from Sigma (catalogue number T6793, T5168, T5595 and anti-phospho-PKCζ (Thr410/403; catalogue number 9378) was obtained from Cell Signaling. DAPI (catalogue number sc-59398), anti-PKCζ (sc-20965), anti-Par6 (catalogue number sc-14405), anti-PC-1 (catalogue number sc-13054), anti-His (catalogue number sc-8036) and anti-β-catenin (catalogue number sc-495) were obtained from Santa Cruz. Anti-HA from Roche (catalogue number 11824800). Anti-Par3 was obtained from Millipore (catalogue number 07–330, diluted at 1:300 for immunofluorescence (IF) and at 1:1,000 for western blot). Anti-E-cadherin was obtained from Invitrogen (catalogue number 131900). Anti-Giantin (catalogue number P08-114C) and anti-pericentrin (catalogue number PRB-432C) were obtained from Covance. Anti-ezrin was obtained from Millipore (97–130, 1:100); anti-occludin from Invitrogen (71–1500, 1:500); and anti-beta-catenin (C2707, 1:500), anti-laminin (9393, 1:500) and anti-CF from Sigma (C2556, 1:500). Rhodamine conjugated-DBA was obtained from Vector Laboratories (cat RL-1032). All primary antibodies were diluted according to the manufacturer’s instructions unless otherwise stated. HRP-conjugated secondary antibodies (GE Healthcare) were diluted at 1:7,000 and detection was made with an ECL system (Amersham).

Analysis of Pkd1 mutant mice. The Pkd1ΔC/mice model has been previously generated and carries loxP sites flanking the two exons of the Pkd1 gene (exons 45 and 46).32. Timed pregnant mice were obtained by overnight crossing wt or Pkd1ΔC/ΔC mice in 100% Bl6/57, and checking for vaginal plugs the next morning. The presence of a vaginal plug was defined as gestational day E0.5 (embryonic day 0.5). All embryos were collected at the defined embryonic stages (between E13.5 and E16.5) and fixed in 4% paraformaldehyde (PFA) in ice-cold methanol, cut in 100–150-μm sections and blocked in 5% low-melting agarose, cut in 100–150-μm sections and stained with DAPI.

Evaluation of tubular diameter in vivo. For the evaluation of the number of cells/tubular cross-section, 12 μm sections of E13.5, 15.5, 16.5 or P1 kidneys were stained with the DBA marker and the nuclear marker DAPI. Nuclei of circle-shaped kidney DBA-positive tubules were counted, excluding from analysis the oval-shaped tubules that have values of ovality at the ImageJ software <0.995 (that is, tubules that are not perpendicularly cross-sectioned). We performed two independent experiments for E13.5, E15.5 and E16.5 live (based on heart beating) littermate embryos, counting 100 wt and 100 mutant tubules for each experiment, as well as for P1 wt kidneys. Whenever the phenotype of the embryos was not overt, the analyses were performed in a blinded manner. For the evaluation of circumference and diameter, for round-shaped tubules, a circumference was tracked going through the centre of all the nuclei of the tubular section; the measure of the circumference and of the related Feret’s diameter was quantified by ImageJ software.

Evaluation of cellular orientation in vivo. For the evaluation of cell elongation and orientation, E13.5 embryos were fixed on in ice-cold methanol, included in 5% low-melting agarose, cut in 100–150-μm sections with a vibratome and stained with DBA, anti-E-cadherin, anti-PKCζ antibodies and DAPI. Z-stacks of renal
tubules were captured every 1 μm, using UltraView spinning disk confocal microscopy (PerkinElmer) with Volocity software. For the evaluation of mediolateral cell movements, cell tracks were tracked using the automated Magnification Assistant tool (Photoshop), on E-cadherin staining in sections taken two frames below (basal to) PKC staining. Only cells in the image where E-cadherin staining outlined the entire cell were used for calculations (cells on the edges that had discontinuous E-cadherin staining were not measured). For all other cells, the length (longest axis) to width (shortest axis) ratio were calculated. Only cells for which the ratio was >1.2 were considered elongated. Those that have a lower ratio (between 1 and 1.2) are depicted in grey and are not considered elongated. For all the others, the angle between the longest axis of the cell and the longitudinal axis of the tubule were calculated using ImageJ software.

Apoptosis and proliferation in vivo. To monitor apoptosis, crystalloid kidney sections of 12 μm were stained with TUNEL and the process was carried out using the DeadEnd Fluorometric TUNEL System Kit (Promega), according to the manufacturer’s protocol. Sections were stained for DBA and were used at a dilution of 1:100 (Vector Laboratories). For proliferation, sections were permeabilized in TritonX-100 blocked with PBS-BSA 3% and normal goat serum 10% at room temperature for 1 h, incubated overnight with the antibody anti-Ki67 or anti-Ph3 at 4 °C, washed and incubated with secondary antibodies (Alexa Fluor-conjugated secondary antibodies from Invitrogen–Molecular Probes), and DBA was used at a dilution of 1:100. The ProLong Antifade kit (Invitrogen–Molecular Probes) was used for mounting the samples.

Wound-healing assays. MDCK type-II and MEF cells were grown on coverslips as high-density monolayers, wounded using a 200-μl pipette tip and allowed to migrate for 3 h (unless otherwise indicated).

For IF studies, paraformaldehyde or methanol-fixed cells were washed, permeabilized in PBS/0.5% Triton X-100 and blocked (PBS, 3% BSA). Primary antibody was applied (all antibodies were diluted to 1:100 in blocking and incubated for 1 h at 37 °C) followed by washes and incubation with secondary antibodies (Alexa Fluor-conjugated secondary antibodies from Invitrogen–Molecular Probes). The ProLong Antifade kit (Invitrogen–Molecular Probes) was used for mounting the samples. Digital images of representative fields were captured using a Zeiss Axiovert fluorescence microscope or UltraView spinning disk confocal microscope (PerkinElmer) equipped with a Plan Apochromat 63X/1.4 oil-immersion objective and using the UltraView ERS acquisition software. For time-lapse studies, cells on the wound edge were tracked using the public domain ImageJ programme (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/iij/). The rate of unidirectional movement was determined following a minimum of ten different cell trajectories from nine movies of three independent experiments (for a total of 102 cells) and measuring the angle formed by the cell trajectories and the perpendicular movement to the wound edge.

Transfection and cell sorting. MDCK cells were transiently transfected using Lipofectamine 2000 (Invitrogen). For IP studies, cells were collected at 4 °C in lysis buffer, composed of 150 mM NaCl, 20 mM NaP, 10% glycerol (pH 7.2) supplemented with phosphatase inhibitors (200 mM glycerol phosphate, 200 mM Na ortho-ascorbate and 500 mM Na fluoride), protease inhibitor cocktail (Complete, Roche) and Triton X-100 as indicated. For cell-sorting experiments, parental MDCK type-II cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions by using 12 μg total DNA. For cell sorting, transfections were performed using a construct expressing green fluorescent protein (pEGFP-N1, Clontech) as a marker for cell sorting in combination with the PAR3 ORF (Fig. 1a, constructs see Fig. 1). The following primers were used for PCR: forward 5′-GTC TGT GGG GGT TTT GGG AGA GAG G-3′, reverse 5′-918-AGACAGACUGGUAGCAGUAUU-3′. For the distribution of cell orientation in the developing tubules (Figs 1e and 6c).

cleaned by centrifugation. GST-fused protein supernatants were incubated at 4 °C with glutathione beads. Beads were centrifuged, washed and incubated with HIS-fused PC-1 supernatants for 3 h at 4 °C (160 μl of volume of this supernatant was used in immunoblotting as input). After centrifuging and washing the beads, proteins were solubilized in Laemmli buffer and analysed by immunoblotting. Full images of western blotting s are shown in Supplementary Fig. 55.

**PAR3 silencing.** Control non-targeting short interfering RNA pool (catalogue number D-001206-13-20) and three short interfering RNA targeting PAR3 (Par3#1: 5′-AGAGCAGUGUGUGCCAGAUUUU-3′; Par3#2: 5′-GUGAAUAUGGUGCCAGCggCUU-3′; Par3#3: 5′-GGUGAAUUUGGCUCCAGCUU-3′), as well as scramble controls were obtained from Thermo Scientific–Dharmacon. To transiently silence Par3, we used Lipofectamine (Invitrogen) transfection, following the manufacturer’s directions.

**Par3-mutant generation and analysis.** HoxB7Cre mice were bred to mice carrying a mutant allele of Par3 (ref. 26). HoXB7Cre;Par3+/− males were crossed to females that were homozygous for an allele of Par3 in which the third coding exon was flanked by loxp sites (Par3flp/flp). Noon of the day of vaginal plug was considered embryonic day 0.5. Litters (half females and half males) were collected at various timepoints for mutant analysis and genotyped by PCR. For Cre-recombinease, the following primers were used: HoXB7Cre: forward 5′-GGT CAC GTG GTC AGA AGA GG-3′, HoXB7Cre: reverse 5′-CTC ATC ATC CTT GTC TGC CAA-3′. Presence of HoXB7Cre results in a 400-bp product. For Par3, the following primers were used: −423F, 5′-AGG CTA GCG TGT GTG ATT TGA GAC C-3′; −359R, 5′-TTC CTT GCT GGC GCC TCA TCC AGT C-3′; and +582F, 5′-GTC TGT GGG GTG TTT GGA AGA G-3′, resulting in products of 371 bp (flox), 292 bp (null) and 265 bp (wt) when resolved on a 3% agarose gel. Mutant embryos (HoXB7Cre;Par3flp/flp−/−) were compared with littermates either with no Cre (Par3−/−) or no null allele (HoXB7CrePar3flp/flp−/−). All animals for these studies were housed, maintained and used according to protocols approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center (animal protocol number 1048-06-01).

**Statistical analysis.** Student’s t-test or one-way analysis of variance was applied to establish differences between means as indicated in the figure legends. Multiple comparisons were carried out using the Bonferroni parameter. Non-parametric Mann–Whitney test was used for the distribution of the angles of migration (Fig. 2b and Supplementary Fig. S3) and for the distribution of cell orientation in the developing tubules (Figs 1e and 6c).

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Acknowledgements
We are grateful to other members of the lab Boletta, and to L. Feltri, T. Watnick and A. Mondino for help in manuscript structuring/writing; to Dr. T. Pawson for providing Par3 constructs; to T. Hirose for providing the Par3-mutant mice; and to Dr. T. Huber for the Par3 GST–PDZ domains. This work was supported by Telethon-Italy (TCR05007 and GGP12183) to A.B. and by the NIH (1R01DK09505) to T.C. and H.R. A.B. is an Associate Telethon Scientist.

Author contributions
M.C. designed and performed the experiments, interpreted them, wrote the manuscript. M.B. designed and performed the initial in-vitro studies on polarized migration. M.Ch. performed the crossings for the Pkd1 mutants, collected and analysed data. H.R. performed the crossings for Par3 mutants, collected and analysed samples. I.R. performed proliferation and apoptosis studies in vivo in Pkd1 mutants and analysed data. M.H. performed the transfection and sorting experiments and analysed data. T.C. supervised the work on Par3 mutants generation and analysis, and critically read the manuscript. A.B. designed the studies, supervised the work and collaborations, and wrote the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/ncomms.

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Castelli, M. et al. Polycystin-1 binds Par3/aPKC and controls convergent extension during renal tubular morphogenesis. Nat. Commun. 4:2658 doi: 10.1038/ncomms3658 (2013).