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Polycystin-1 and polycystin-2 regulate the cell cycle through the helix–loop–helix inhibitor Id2

Xiaogang Li[1], Ying Luo[1], Patrick G. Starremans[1], Coleen A. McNamara[2], York Pei[3] and Jing Zhou[1,4]

Autosomal-dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease and is characterized by progressive cyst formation and ultimate loss of renal function. Increased cell proliferation is a key feature of the disease. Here, we show that the ADPKD protein polycystin-2 (PC2) regulates the cell cycle through direct interaction with Id2, a member of the helix–loop–helix (HLH) protein family that is known to regulate cell proliferation and differentiation. Id2 expression suppresses the induction of a cyclin-dependent kinase inhibitor, p21, by either polycystin-1 (PC1) or PC2. The PC2–Id2 interaction is regulated by PC1-dependent phosphorylation of PC2. Enhanced Id2 nuclear localization is seen in human and mouse cystic kidneys. Inhibition of Id2 expression by RNA interference corrects the hyperproliferative phenotype of PC1 mutant cells. We propose that Id2 has a crucial role in cell-cycle regulation that is mediated by PC1 and PC2.

Cysts in autosomal-dominant polycystic kidney disease (ADPKD) originate from various kidney tubular segments and other epithelial ductal structures in the liver and pancreas[1]. They are lined by a single layer of epithelium that is characterized by increased cellular proliferation and decreased differentiation[2,3]. Other features include abnormalities in gene expression, cell polarity, fluid secretion, apoptosis and extracellular matrix[3–6]. The molecular mechanism of cyst formation remains incomplete. Complications include cardiac valvular abnormalities and intracranial aneurysms. Mutations in *PKD1* or *PKD2*, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively, account for almost all ADPKD cases. PC1 encodes an integral membrane glycoprotein[7,8] that acts as a G-protein-coupled receptor[9,10] and is believed to be important for cell–cell and cell–matrix interactions. PC2 is a Ca^{2+}-permeable cation channel[11–14], the C-terminus of which contains a calcium-binding EF-hand and a coiled-coil domain that mediates its interaction with PC1 (refs 15–17). PC2 interacts with tropomyosin-1 and troponin *in vitro*[18,19]. PC1 and PC2 are found in the plasma membrane and the endoplasmic reticulum[20]. They co-express in the cilia of renal epithelia and mediate mechanosensation as a receptor-channel complex[21].

PC1 and PC2 are likely to function in a common pathway because mutations in either gene, whether in mice or humans, cause strikingly similar phenotypes. *Pc1*- and *Pc2*-knockout mice have defective epithelial differentiation and maturation in the kidney and pancreas and die perinatally[22–24], indicating that they are required for organ development. Whereas PC2 seems to negatively regulate the G-protein activation function of PC1, PC1 can activate the PC2 channel[21,25]. Therefore, mutations in either *PKD1* or *PKD2* may disrupt the polycystin complex and subsequently deregulate the polycystin-mediated signal transduction pathways that normally control the differentiation and proliferation of kidney epithelial cells.

Id2 is a member of a helix–loop–helix (HLH) family of transcription factors that promote cellular growth and inhibit differentiation[26–29]. The Id proteins act as dominant-negative inhibitors of basic HLH proteins, inhibiting DNA binding and subsequent activation of transcription.

We show here that PC2, through direct interaction with Id2, regulates the nuclear translocation of Id2 and modulates the cell cycle through the Id2–p21–Cdk2 pathway. Using PC1 stable expression cell lines and genetically engineered PC1 mutant cells, we demonstrated that Id2–PC2 interactions require PC1-dependent serine phosphorylation of PC2. *PKD1* mutations result in abnormalities in the cell cycle and inhibition of *Id2* mRNA expression by RNA interference (RNAi) reverses the cell-cycle profile to normal.

RESULTS

PC2 overexpression or silencing alters the cell-cycle profile of HEK293T cells

To evaluate the role of PC2 on cell proliferation, we measured the proliferation rate of a stable tetracycline-inducible PC2-overexpressing cell line, PC2S1, and found that the induced PC2S1 cells proliferate approximately 60% slower than non-induced PC2S1 cells (Fig. 1a). A similar proliferation rate was observed in mouse inner-medullar collecting duct (IMCD) cells overexpressing PC2 (data not shown). To define the mechanisms by which PC2 exerts these effects, we tested whether the inhibition of proliferation was cell-cycle-dependent. About 74% of the

[1]Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA. [2]Cardiovascular Division, Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA. [3]Division of Nephrology, Department of Medicine, Toronto Hospital and University of Toronto, Toronto, Ontario M5G2C4, Canada.
[4]Correspondence should be addressed to J.Z. (e-mail: zhou@rics.bwh.harvard.edu)

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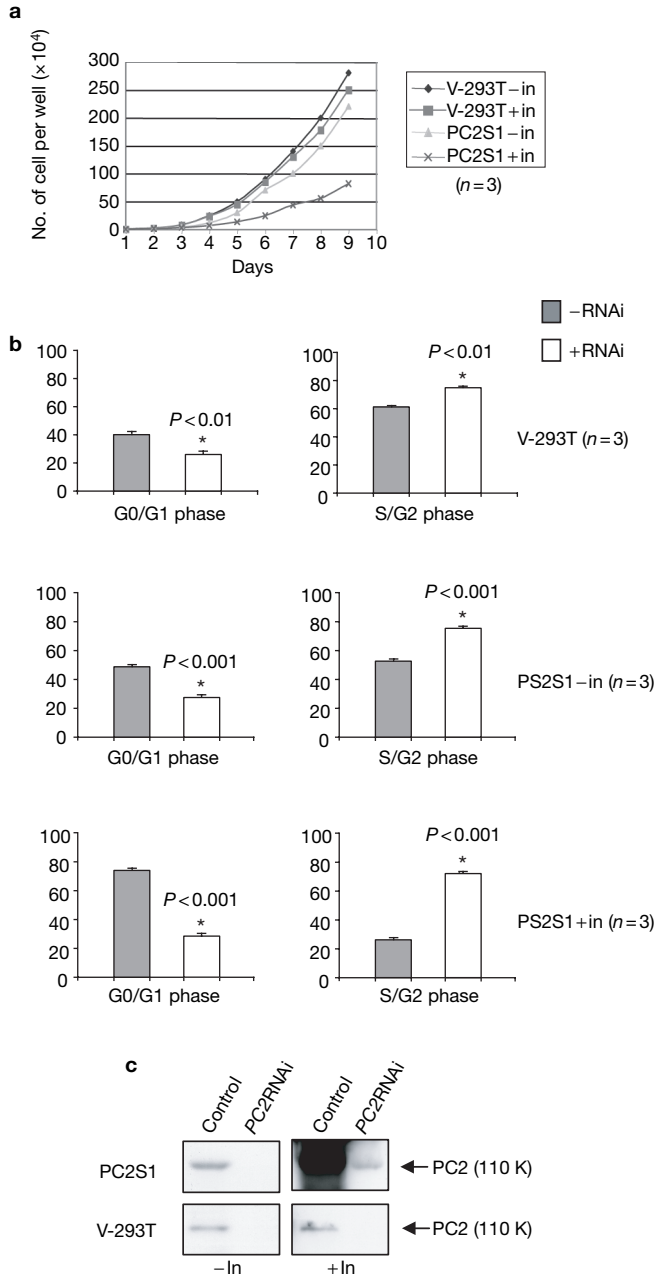

Figure 1 PC2 expression regulates the cell cycle in 293T cells. (**a**) PC2 overexpression results in slow growth. Growth curves (average of three experiments) of cells stably transfected with vector (V-293T) or wild-type PC2 (PC2S1) with (+in) or without (–in) tetracycline induction are shown. (**b**) Inhibition of endogenous or recombinant PC2 by RNA interference (RNAi) releases the cell-cycle arrest at the G0/G1 phase due to PC2 overexpression and promotes cell-cycle progression. *P* value indicates the significance of differences in the percentage of cells with versus without PC2 RNAi at a given cell-cycle phase. Error bars indicate SEM. (**c**) Western blot analyses show that PC2 RNAi greatly reduces the levels of endogenous (left panels) and overexpressed PC2 (upper right panel). Control, RNAi vector alone; +in, induced; –in, uninduced; K, relative molecular mass in thousands.

induced PC2S1 cells were arrested in the G0/G1 phase. By contrast, only ~40% of the control empty vector stably transfected 293T cells (tetracycline-induced and non-induced) and ~48% of non-induced PC2S1 cells accumulated in the G0/G1 phase (Fig. 1b), indicating that PC2 overexpression arrests the cell cycle. To determine whether inhibition of PC2 expression can release the cell-cycle block, we used the DNA vector-based RNAi technology to suppress *PC2* mRNA expression in PC2S1 and control 293T cells. Transfection of a green fluorescent protein (GFP)-tagged PC2 RNAi construct (pBS/U6-EGFP/PC2) inhibited endogenous PC2 expression in control 293T cells. This inhibition was most prominent in the PC2-overexpressing cells, PC2S1 (Fig. 1c). PC2 silencing released the G0/G1 cell-cycle arrest in PC2S1 cells (Fig. 1b) and promoted cell-cycle progression to S and G2 phases in PC2S1 and the control 293T cells (Fig. 1b).

Identification of PC2 downstream signalling partners involved in cell-cycle regulation

To identify PC2 downstream signal transduction in cell-cycle regulation, we performed yeast two-hybrid screening[30]. The entire carboxy-terminal intracellular domain of human PC2 was used to screen a human fetal kidney cDNA library. PC2 interacted with Id2, a member of the HLH protein family that acts as a dominant-negative regulator of HLH proteins that are important for cell proliferation and cell differentiation. Id2 is also a target of β-catenin that can be stabilized by the PC1 C-terminal tail[31].

To verify the specificity of this interaction and to map the PC2-interacting domain within Id2, we made a series of deletion constructs of Id2 (Fig. 2a) and tested their interaction with PC2 in the yeast two-hybrid system. The Id2 deletion construct containing the HLH region interacted with the PC2 C-terminal tail as efficiently as did the full-length Id2 (Fig. 2a). Deletion constructs containing either the amino-terminal or the C-terminal domain of Id2 did not interact with PC2, indicating that the PC2 binding domain is located within the HLH region of Id2. Yeast co-transformation of the full-length Id2 with the PC2 C-terminal tail or with a PC2 C-terminal tail deletion construct (Fig. 2b) indicated that the Id2 binding domain is located within residues 741–968 of PC2.

To verify the PC2–Id2 association in mammalian cells, we immunoprecipitated 293T cells that had been transfected with Myc-tagged full-length PC2 or PC2-S812A — the serine of which at 812 was replaced with alanine — with anti-Myc or anti-Id2 antibody (Fig. 2c). Full-length PC2 could be co-immunoprecipitated by endogenous Id2. Conversely, endogenous Id2 could be co-immunoprecipitated with full-length PC2 (Fig. 2c). Myc-tagged PC2 mutant (PC2-S812A) failed to be co-immunoprecipitated with Id2, and endogenous Id2 could not immunoprecipitate PC2 that lacked its C-terminal tail (Fig. 2d). These results indicated that the C-terminal tail and the serine at position 812 of PC2 are necessary for Id2–PC2 interactions.

PC2 sequesters Id2 in the cytoplasm

To confirm the functional relationship of the PC2 and Id2 interaction *in vivo*, we determined the localization of PC2 and Id2 in 293T cells. Although endogenous Id2 localized in both the nucleus and cytosol in the parental 293T and IMCD cells (data not shown), it localized with PC2 in the cytoplasm (Fig. 3a) of PC2S1 cells that were stably overexpressing Myc-tagged PC2, indicating that PC2 expression may prevent Id2 from entering the nucleus.

Data from western blot analyses of Id2-transfected PC2S1 cells corroborated the immunofluorescence results (Fig. 3b). Induction of PC2 in PC2S1 cells led to more Flag-tagged Id2 in the cytosolic fraction than in the nuclear fraction. Knockdown of *PC2* in the PC2S1 cells by RNAi resulted in the translocation of Id2 into the nucleus (Fig. 3c).

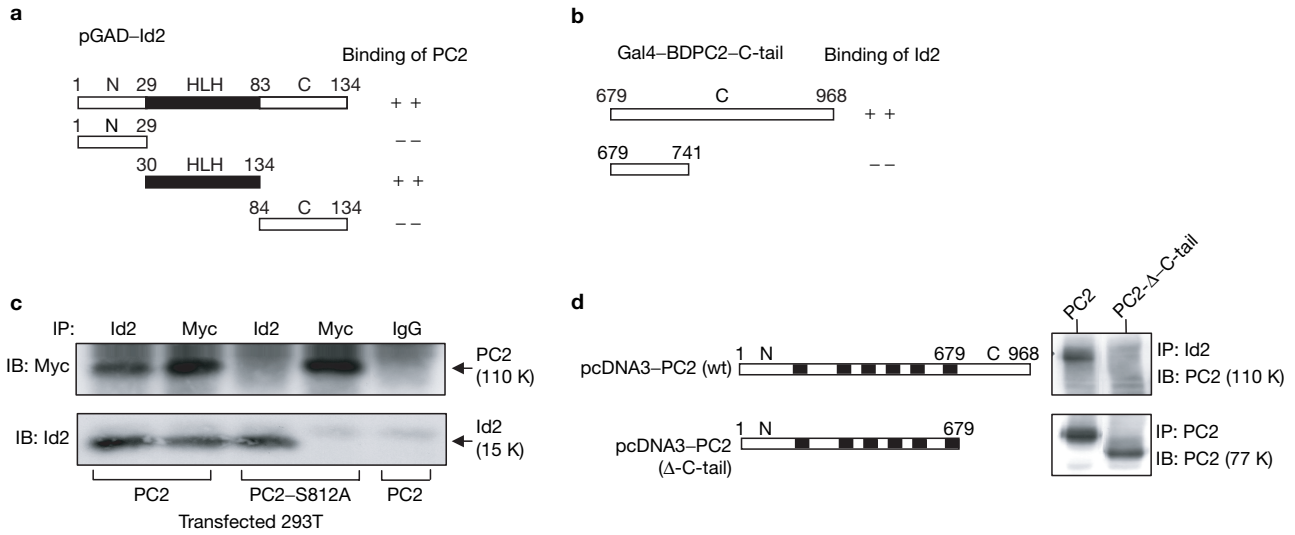

Figure 2 PC2 interacts with Id2. (**a**, **b**) Interaction between Id2 and PC2 carboxy-terminal-tail in yeast. N, N-terminal; C, C-terminal; HLH, helix–loop–helix region; +/–, binding strengths. (**c**, **d**) Endogenous Id2 associates with transiently transfected full-length wild-type (wt) PC2–Myc but not mutant PC2–S812A (**c**) nor PC2-Δ–C-tail (**d**) in 293T cells. Immunoglobulin G was used as the control. A faint band just above the Id2 band is non-specific, as reported previously[40]. The numbers refer to residues in Id2 or PC2. IP, immunoprecipitation, IB, immunoblot; K, relative molecular mass in thousands.

Nuclear localization of Id2 in polycystic kidneys from patients with *PKD1* and *PKD2* mutations

To determine whether Id2 could contribute to the abnormal cell proliferation and differentiation in ADPKD, we examined Id2 expression patterns in normal and cystic kidneys from patients with ADPKD (Fig. 4a). By confocal microscopic analyses, we found increased Id2 expression and nuclear translocation in cyst-lining epithelia in the kidneys from patients with either *PKD1* or *PKD2* mutations. This is in contrast to the normal kidney, in which a low level of Id2 expression was detected, primarily in the cytosol. The specificity of Id2 antibody was confirmed by using *Id2* knockout tissues (see Supplementary Information, Fig. S1). The expression of Id2 in normal adult kidney and the upregulation in ADPKD kidney were confirmed by immunoprecipitation followed by western blotting (Fig. 4a, inset). These results provided us with a clue to investigate the relationship between PC1 and PC2–Id2.

Function of Id2 in PC1-overexpressing cells

Bhunia *et al.*[32] have reported that PC1-overexpressing cells cause cell-cycle arrest. To investigate the role of Id2 in this process, we examined Id2 localization in the PC1-overexpressing cells, PC1S[20]. Transiently expressed Id2 was localized in the cytosol of the induced PC1S cells (Fig. 4b) but was expressed in the nucleus and cytosol of the non-induced PC1S cells (Fig. 4c). These results were indistinguishable from those in PC2-overexpressing cells. To understand the mechanism underlying the Id2 cytosolic retention in PC1-overexpressing cells, we explored a possible role of PC2. Because the PC2 protein level seemed to be unchanged (Fig. 4d, right panel), we investigated the phosphorylation status of PC2 and its interaction with Id2 in the PC1-overexpressing cells. Serine-phosphorylated PC2 was significantly increased in the PC1-overexpressing cells (Fig. 4d). A greater amount of PC2 was co-immunoprecipitated by Id2 in the PC1-overexpressing cells, raising the possibility that Id2 interacts with serine-phosphorylated PC2 (Fig. 4d). As PC2 overexpression facilitates PC2–Id2 interactions, we next examined the phosphorylation of PC2 in the PC2S1 cells. We found an increased pool of serine-phosphorylated PC2 in induced PC2-overexpressing cells, although not all PC2 protein was serine phosphorylated (Fig. 4e).

Id2–p21–Cdk2 pathway is regulated by PC2

PC2 overexpression inhibits the transition of the cell cycle from G0/G1 into the S phase (Fig. 1b). Because Id2 is known to regulate the cell cycle through p21 and the cyclin-dependent kinase (CDK) Cdk2 (refs 33, 34), we examined whether PC2 exerted its effects on cell-cycle regulation by altering the activity of the CDKs and/or CDK inhibitors, especially p21 and Cdk2, which are related to Id2 function.

We tested whether PC2 expression results in p21 activation using a luciferase reporter assay in PC2S1 cells. We used PC1 stably transfected cells as a control, as a recent study suggested that PC1 overexpression induces p21 expression and further regulates the cell cycle via direct activation of the JAK–STAT signalling pathway[32]. We found that PC2 overexpression induced about a three-fold increase in luciferase reporter activity (Fig. 5a). Furthermore, PC2-induced p21-luc expression could be inhibited by increasing Id2 expression in the same cells, with or without induced PC2 expression (Fig. 5a). Similar effects were seen in PC1 stable cells (Fig. 5b). These results indicate that the inhibitory effects on p21 by Id2 overexpression completely override PC1- and PC2-induced p21 transcription, indicating that stoichiometry of the protein–protein interactions between Id2 and PC2 is crucially important in regulating the cell cycle.

We next determined the levels of p21 protein, other CDK inhibitors and Cdk2 under PC1 and PC2 overexpression conditions. We found a significant induction of p21 but no change in p16 and p27 protein levels in cells overexpressing PC1 or PC2 (Fig. 5c). PC1 and PC2 overexpression caused a reduction in Cdk2 activity, whereas no effects on protein level were seen (Fig. 5c). This is probably due to an increased recruitment of Cdk2 by p21 (Fig. 5c). These results indicate that PC2 blocks the cell cycle through upregulation of p21 and subsequently inhibits Cdk2 activity but not its expression. A stronger induction of p21 levels by PC2 than by PC1 overexpression was seen, which is

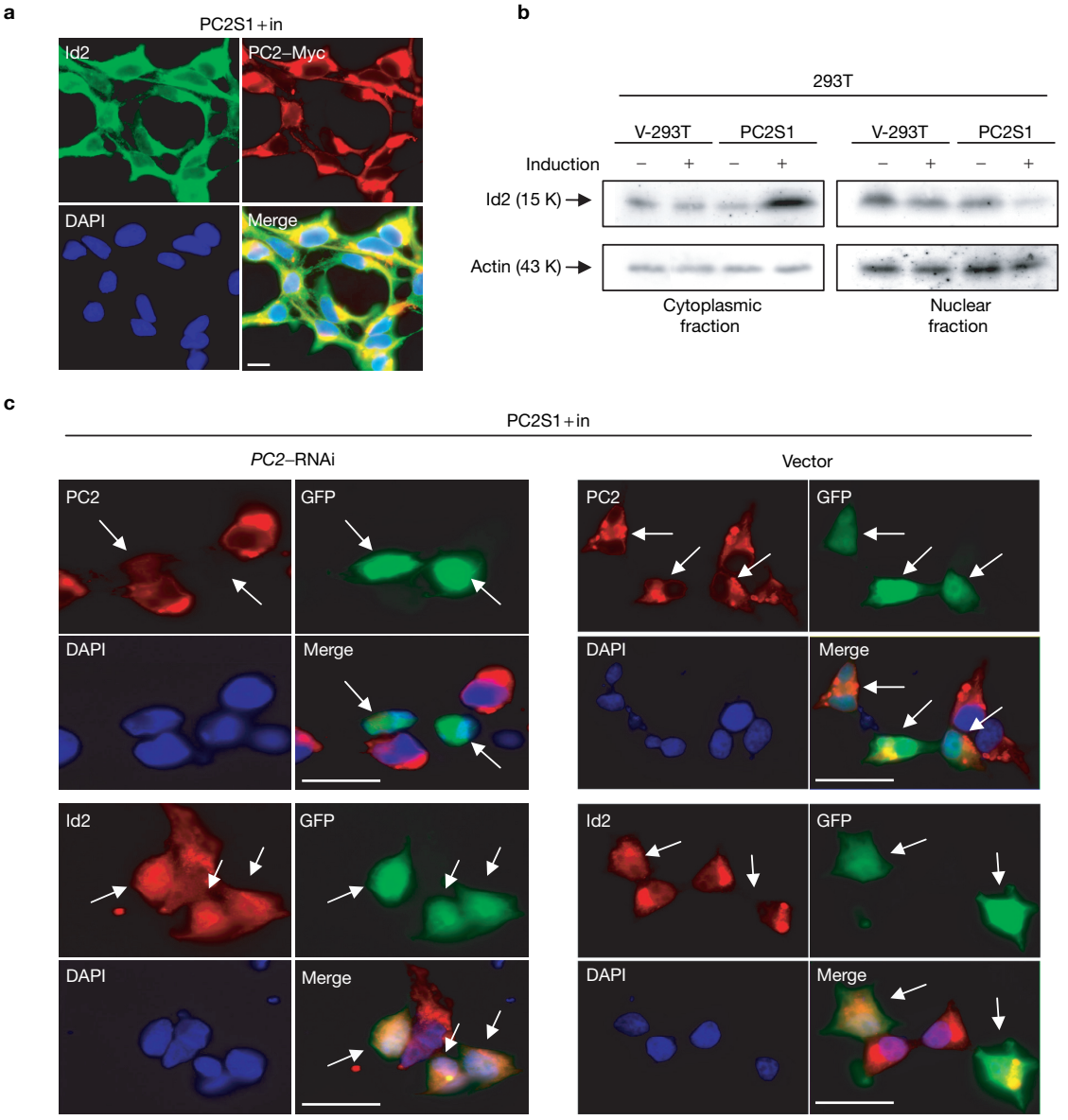

Figure 3 PC2 modulates Id2 nuclear translocation in 293T cells. (**a**) Induction of PC2–Myc (red) expression in PC2S1 cells sequesters endogenous Id2 (green) outside of the nucleus. (**b**) Western blot analyses with anti-Flag antibody confirms that PC2 overexpression sequesters transiently transfected Flag–Id2 in the cytosolic fraction. Actin, loading controls. K, relative molecular mass in thousands. (**c**) Nuclear translocation of endogenous Id2 in induced PC2S1 cells transfected with PC2RNAi but not with RNA interference (RNAi) vector. As the RNAi vector contains a green fluorescent protein cassette, all transfected cells (arrows) are green. Scale bars, 10 μm. +/–, with/without induction.

consistent with the results of our luciferase activity assay. Upregulation of p21 expression was further confirmed at the transcription level by northern blot analysis (Fig. 5d).

PC1 regulates Id2 localization in mouse epithelial kidney cells isolated from *Pkd1*-targeted mice and their normal littermates

Mice with a targeted *Pkd1*$^{null/null}$ or *Pkd1*$^{del34/del34}$ mutation lack functional PC1 and develop severe ADPKD[22,23]. By confocal microscopic analyses, we found greater nuclear translocation of Id2 in cyst-lining epithelia in the *Pkd1*$^{null/null}$ kidneys than that in kidney tubules from their normal littermates (Fig. 6a).

To confirm the immunostaining data, we performed western blot analyses of kidney tissue extracts and nuclear and cytosolic fractions of mouse epithelial kidney (MEK) cells from wild-type and *Pkd1* targeted mice[21–23]. The MEK cells were recently isolated from collecting ducts (positive for *dolichos biflorus agglutinin,* DBA) and proximal tubules (positive for *lotus tetragonolobus,* LTA) of E15.5 kidneys of *Pkd1*$^{del34/del34}$ and *Pkd1*$^{null/null}$ mutants and their wild-type littermates[21–23]. In kidneys from the *Pkd1*$^{null/null}$ mice, p21 was significantly reduced although Id2 expression was unaffected (Fig. 6b). Id2 expression increased in the nuclear fraction and decreased in the cytosolic fraction in the *Pkd1*$^{del34/del34}$ and *Pkd1*$^{null/null}$ cells compared with that in the wild-type cells (Fig. 6c, d). Increased Id2 expression correlated with a reduction in the p21 levels in the nucleus but with an increase of Cdk2 levels in homozygous *Pkd1*$^{null/null}$ cells. A concomitant decrease in the levels of Id2, Cdk2 and an increase in p21 was seen in the cytoplasmic fraction in homozygous *Pkd1*$^{null/null}$ cells. Cdk2 expression is known to promote Id2 phosphorylation and nuclear translocation[33,34].

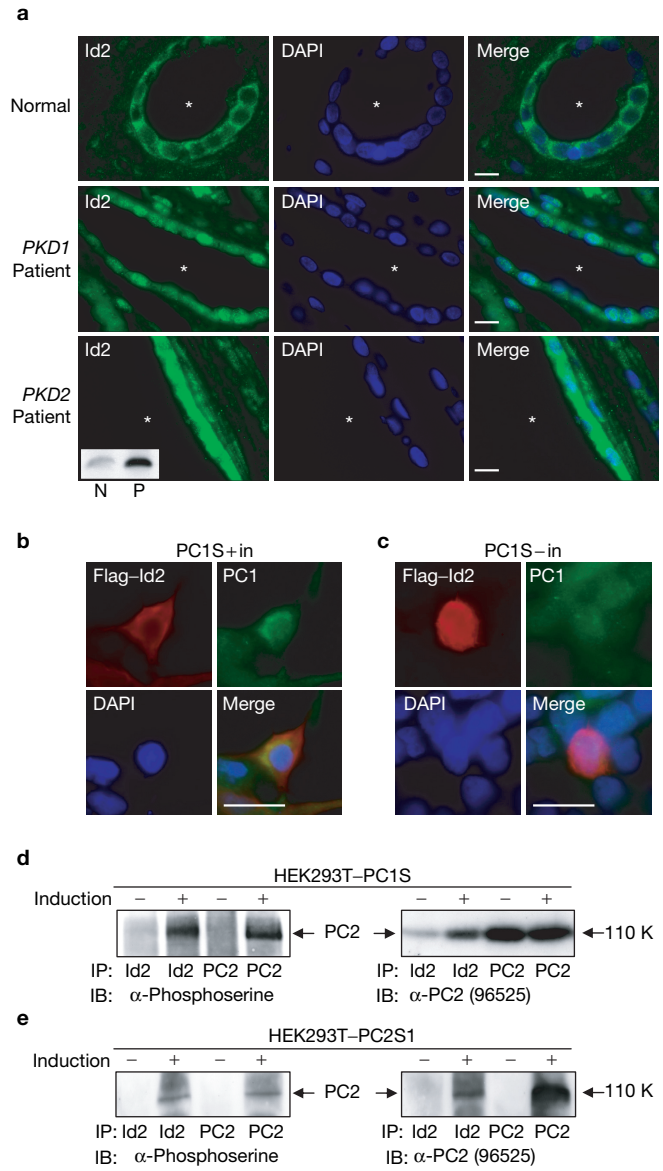

Figure 4 Inter-relationship among Id2, PC2 and PC1. (**a**) Top panel. Id2 in the cytosol of normal human adult kidney tubules. Middle and bottom panels. Increased Id2 nuclear expression in cyst-lining epithelia in patients with *PKD2* or *PKD1* mutations. Inset. Id2 expression in normal (N) and patient (P) kidneys. Original magnification, ×100. * indicates lumen. Scale bars, 10 μm. (**b**, **c**) Induction of PC1 expression in PC1S cells retains transiently transfected Flag–Id2 in the cytosol. PC1 (**d**) and PC2 (**e**) overexpression induces PC2 phosphorylation. Id2 seems to interact with phosphorylated PC2. K, relative molecular mass in thousands.

To study the role of PC2 in the nuclear translocation of Id2 in PC1 mutant cells, we investigated the PC2–Id2 interaction. Unlike in the wild-type MEK cells in which Id2 could co-immunoprecipitate serine-phosphorylated PC2, Id2 failed to pull down PC2 in the MEK cells with either the *Pkd1*$^{del34/del34}$ or *Pkd1*$^{null/null}$ mutation (Fig. 6e). In fact, there is a defect in PC2 serine phosphorylation (Fig. 6e). These results indicate that the interaction between Id2 and PC2 is disrupted in PC1 mutant cells and that PC1 is required for PC2 phosphorylation and PC2–Id2 interactions.

Interactions between Id2, PC2 and the E2A gene product, E47

E47 is a ubiquitous HLH protein that is known to turn on the *p21* gene expression via direct binding to the *p21* promoter[27]. To investigate the effects of E47 on cytosolic sequestration of Id2 by PC2, we performed a set of immunostaining experiments. In PC2 stable (PC2S1) cells without induction or in control vector stable cells (V-293T), transfection of E47 brought Id2 into the nucleus (Fig. 7a), as previously reported in other cell types[34]. PC2 induction sequestered Id2 into the cytosol regardless of the transfection of E47 in PC2S1 cells, but not in control vector stable cells (Fig. 7b), indicating that the PC2–Id2 interactions prohibit E47-mediated nuclear translocation of Id2.

To determine whether E47 interacts with PC2-bound Id2 in wild-type and PC1 mutant cells, we performed immunoprecipitation in the two wild-type and four PC1 mutant cell lines. Although PC2 and Id2 can only be reciprocally co-immunoprecipitated in the two wild-type cell lines, both PC2 and Id2 were present in all the six MEK cell lines (Fig. 7b, left panels). Interestingly, E47 was found in the PC2 pellet, indicating the existence of a PC2–Id2–E47 protein complex. Immunoprecipitation of the supernatant of PC2 precipitation revealed that, E47, although found in all six cell lines, could co-immunoprecipitate Id2 in the four mutant lines, but not in the wild-type cells. This may represent a significant depletion of Id2 by PC2 immunoprecipitation in the two wild-type cell lines. The limited amount of Id2 in the supernatant of PC2 immunoprecipitation was also revealed by Id2 precipitation, which pulled down about five-fold more E47 protein in the four types of mutant cells in which the PC2–Id2 interaction was disrupted compared with the wild-type cells (Fig. 7b, left panels). This indicates that most of the Id2–E47 complexes exist in the absence of PC2–Id2 interactions. The presence of PC2–Id2–E47 complexes was further confirmed by Id2 immunoprecipitation (Fig. 7b, right panels). When PC2 cannot bind Id2 (as in the PC1 mutant cells), interaction with E47 brings Id2 into the nucleus to exert its dominant-negative effects on E47 and other HLH factors that control the transcription of cell-cycle genes, such as *p21*.

Inhibition of Id2 mRNA reverses the cell-cycle profile of polycystic kidney cells

Polycystic kidney cells are less differentiated than normal kidney cells and have an increased expression of proliferative markers, such as c-Myc and proliferative cell nuclear antigen[2,35]. To determine whether the hyperproliferative feature of polycystic kidney cells requires Id2, we transiently suppressed *Id2* gene expression by RNAi in wild-type and homozygous *Pkd1*$^{null/null}$ cells. Transient expression of pBS/U6-EGFP/Id2 greatly inhibited Id2 expression (Fig. 8a). Comparison of the cell-cycle profiles of either DBA- or LTA-positive wild-type MEK cells with those of the respective homozygous *Pkd1*$^{null/null}$ cells revealed that, for both DBA- and LTA-positive cells, 62% and 64% of the wild-type cells, but only 25% and 27% of the homozygous *Pkd1*$^{null/null}$ cells, accumulated in the G0/G1 phase (Fig. 8b, left panel). A majority of the homozygous cells were situated between the S and G2 phases (Fig. 8b, right panel). Inhibition of *Id2* mRNA by RNAi greatly altered the cell-cycle profile in homozygous *Pkd1*$^{null/null}$ mutant cells, resulting in a significant reduction in S to G2 phase entry and a significant increase in cells in the G0/G1 phase (from 25% and 27% to 61% and 63%, respectively) (Fig. 8b).

DISCUSSION

As is the case with several other positive regulators of cell-cycle progression, the function of Id proteins is also regulated by subcellular localization. Here, we report that PC2 interacts with Id2, which in turn

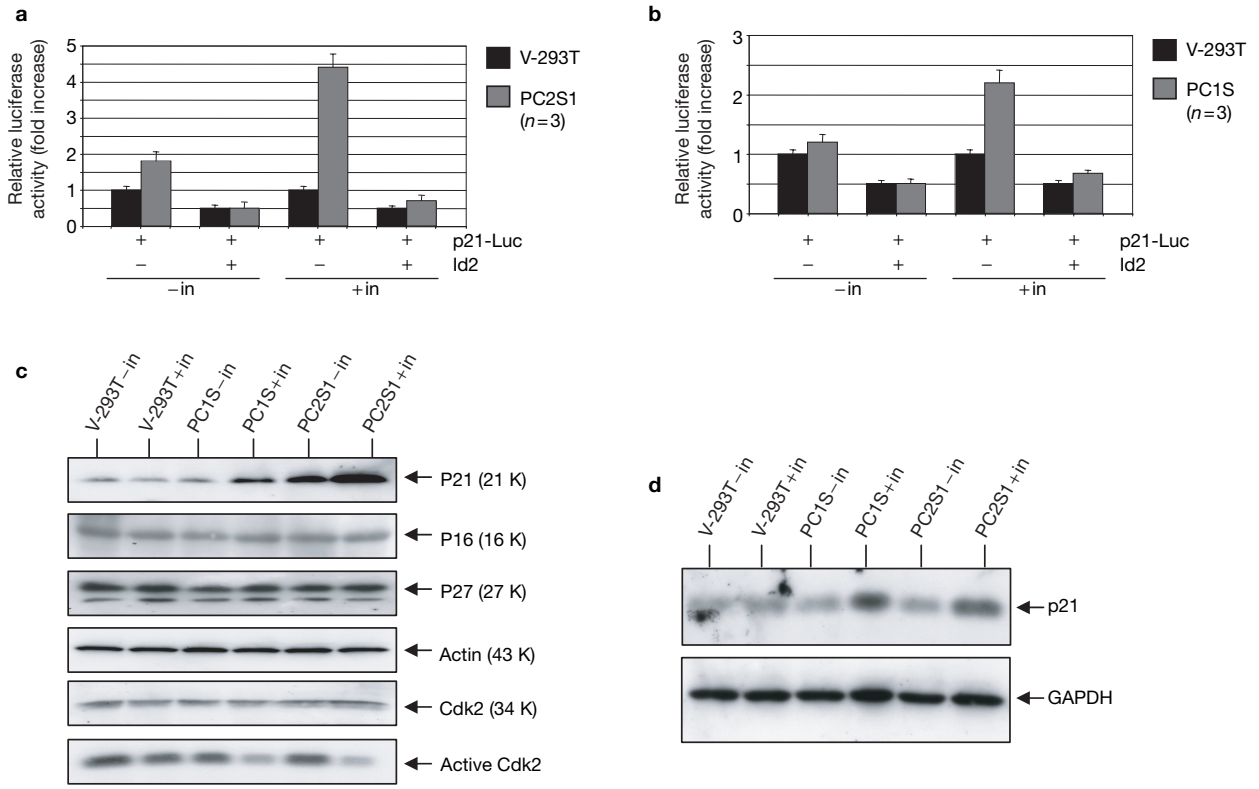

Figure 5 PC2 regulates the Id2–p21–Cdk2 pathway. PC2 (**a**) or PC1 (**b**) stably transfected 293T cells were transfected with p21–luc or p21–luc and Id2. Id2 inhibits PC2- or PC1-induced *p21* promoter activity. The histogram indicates the fold change in relative luciferase activity (RLA) in 293T cells stably transfected with vector (V-293T) or PC1 or PC2. RLA of p21–luc in V-293T cells is expressed as 1. Error bars indicate SEM. Western (**c**) and northern (**d**) blot analyses confirmed the induction of p21 by PC1 or PC2 at the protein and transcript levels. PC1 or PC2 expression induced Cdk2 activity, measured by an *in vitro* histone-1 (H1) phosphorylation assay, but not expression levels. GADPH, glyceraldehyde dehydrogenase phosphate; K, relative molecular mass in thousands.

sequesters Id2 outside the nucleus (Figs 2, 3). Interestingly, PC1-overexpressing cells also retain most of the Id2 proteins in the cytosol, probably by increasing serine phosphorylation of PC2. Serine-phosphorylated PC2 interacts with Id2 (Fig. 4d).

Although overexpression of both PC1 and PC2 increases the amount of serine-phosphorylated PC2, the underlying mechanism may be different: for PC1, it may include the recruitment of a kinase into the PC1–PC2 complex directly or indirectly; for PC2, it may result from an increase in the pool of PC2 that is available for phosphorylation, as only a portion of the PC2 proteins was phosphorylated (Fig. 4e).

Notably, increased Id2 nuclear localization is clearly seen in renal epithelial cells from kidneys of *PKD1* and *PKD2* patients (Fig. 4a) and from *Pkd1* targeted mice (Fig. 6a). How does a *PKD1* mutation promote nuclear translocation of Id2? Our data indicate that abnormal phosphorylation of PC2 in *Pkd1*$^{del34/del34}$ and *Pkd1*$^{null/null}$ cells would cause the disruption of PC2–Id2 interactions (Fig. 6d). Thus, the balance between Id2 expression, localization, and the protein levels and phosphorylation status of PC2 plays a crucial role in the subcellular distribution of Id2, signalling into the nucleus and the control of cell proliferation and differentiation. The CDK inhibitor p21 is induced by PC1 overexpression and is downregulated in cystic kidneys[32,36,37]. Here, we provide evidence that the p21–Cdk2 pathway is regulated by the PC2–Id2 interaction (Fig. 5). Nuclear Id2 can repress transcription of p21 and promote cell-cycle progression in kidney cells. We have also observed increased Id2 expression in adult cystic kidneys (Fig. 4a), which may contribute to the less differentiated phenotype of ADPKD kidneys. An important role of Id2 in cell-cycle regulation in ADPKD is demonstrated by the RNAi experiments. Inhibition of Id2 expression in the PC1 mutant cells that are hyperproliferative strikingly reverses their cell-cycle profile to normal (Fig. 8b). Little is known about the function of Id proteins in the kidney. Our data provide direct evidence that increased nuclear Id2 expression contributes to abnormal epithelial proliferation or differentiation in the kidney (Fig. 8b, c). PC2 also interacts with other members of the Id protein family (data not shown). These interactions may regulate Id-dependent cell proliferation and differentiation in different cell types.

The PC2–Id2 pathway links to several other important signalling proteins in addition to the p21 pathway, some of which have been suspected to play a role in the pathogenesis of ADPKD. These proteins, which include β-catenin[38], E-cadherin[39], c-Myc[40] and retinoblastoma (Rb) protein, may also contribute to the hyperproliferative phenotype in cyst-lining epithelia. β-Catenin, which has been found to associate with PC1, can activate transcription of Id2 (ref. 38), probably through c-Myc[41], which is overexpressed in cystic kidneys[35,42]. The HLH domain of Id2 that binds to PC2 also binds to Rb. However, c-Myc–Id2 activation may bypass the growth suppression function of Rb.

The Id proteins lack a nuclear-localization signal. They are transported to the nucleus by E47 and other E-proteins[34,43]. Id2 antagonizes E47-dependent modulation of gene expression, such as that of *p21* (ref. 27),

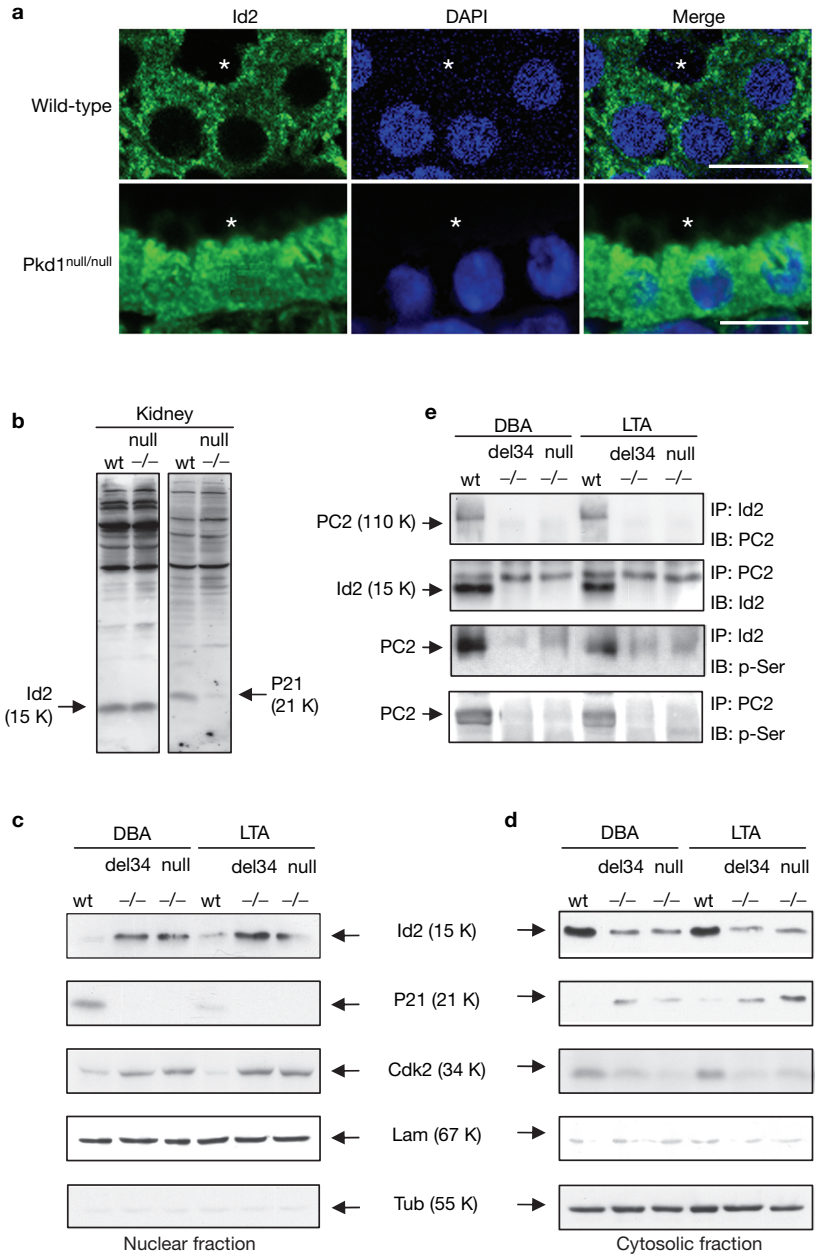

Figure 6 PC1 mutation disrupts the PC2–Id2 interaction. (**a**) Nuclear Id2 localization in cysts of *Pkd1*$^{null/null}$ kidneys but cytosolic localization in tubules of the wild-type littermate mouse at E18.5. Original magnification, ×100. * indicates lumen. Scale bars, 10 μm. (**b**) Western blot analyses of kidneys from newborn wild-type and *Pkd1*$^{null/null}$ mice. (**c**, **d**) Immunoblot of endogenous Id2, p21 and Cdk2 in nuclear (**c**) and cytosolic (**d**) fractions of the wild-type and *Pkd1*$^{null/null}$ kidney epithelial cells positive for either a collecting (*dolichos biflorus agglutinin*, DBA) or a proximal (*lotus tetragonolobus*, LTA) tubule marker. Nuclear Id2 is clearly increased in both DBA- and LTA-positive *Pkd1*$^{null/null}$ cells. Lamin-B (Lam) and tubulin (Tub) mark the nuclear and cytosolic fractions and equal loading. (**e**) Disrupted PC2–Id2 interactions in *Pkd1*$^{del34/del34}$ and *Pkd1*$^{null/null}$ cells. Id2 co-precipitated serine-phosphorylated PC2 in wild-type but not in PC1 mutant cells. K, relative molecular mass in thousands.

and E-cadherin[39] further modulates the cell cycle and cell differentiation. Prevention of E47-mediated nuclear translocation of Id2 is a possible downstream mechanism by which PC2 controls the cell cycle. We have shown here that the PC2 protein complex contains Id2 and E47, and that the PC2–Id2 interaction regulates the Id2–E47 protein complex (Fig. 7). This conclusion was based on two observations: despite its nuclear location in the non-induced cells, Id2 was located in the cytosol of the induced PC2S1 cells that were transfected with E47 (Fig. 7a); and both Id2 and E47 were found in the pellet of PC2 immunoprecipitation, indicating that they are in the PC2 protein complex. The abundant Id2–E47 complex was only found in the supernatant of PC2 immunoprecipitation in PC1 mutant MEK cells, where the PC2–Id2 interaction was disrupted. These results were further confirmed by Id2 immunoprecipitation (Fig. 7b). It remains possible, however, that the interactions between Id2–E47 with PC2 are modulated by many factors in addition to the serine-phosphorylation status of PC2. Previous studies have shown that the phosphorylation state of Id2 regulates Id2 partner selectivity and cellular localization[34].

The role of Id2 in cell proliferation and differentiation has recently been linked to Smad4-dependent transforming growth factor-β (TGF-β)

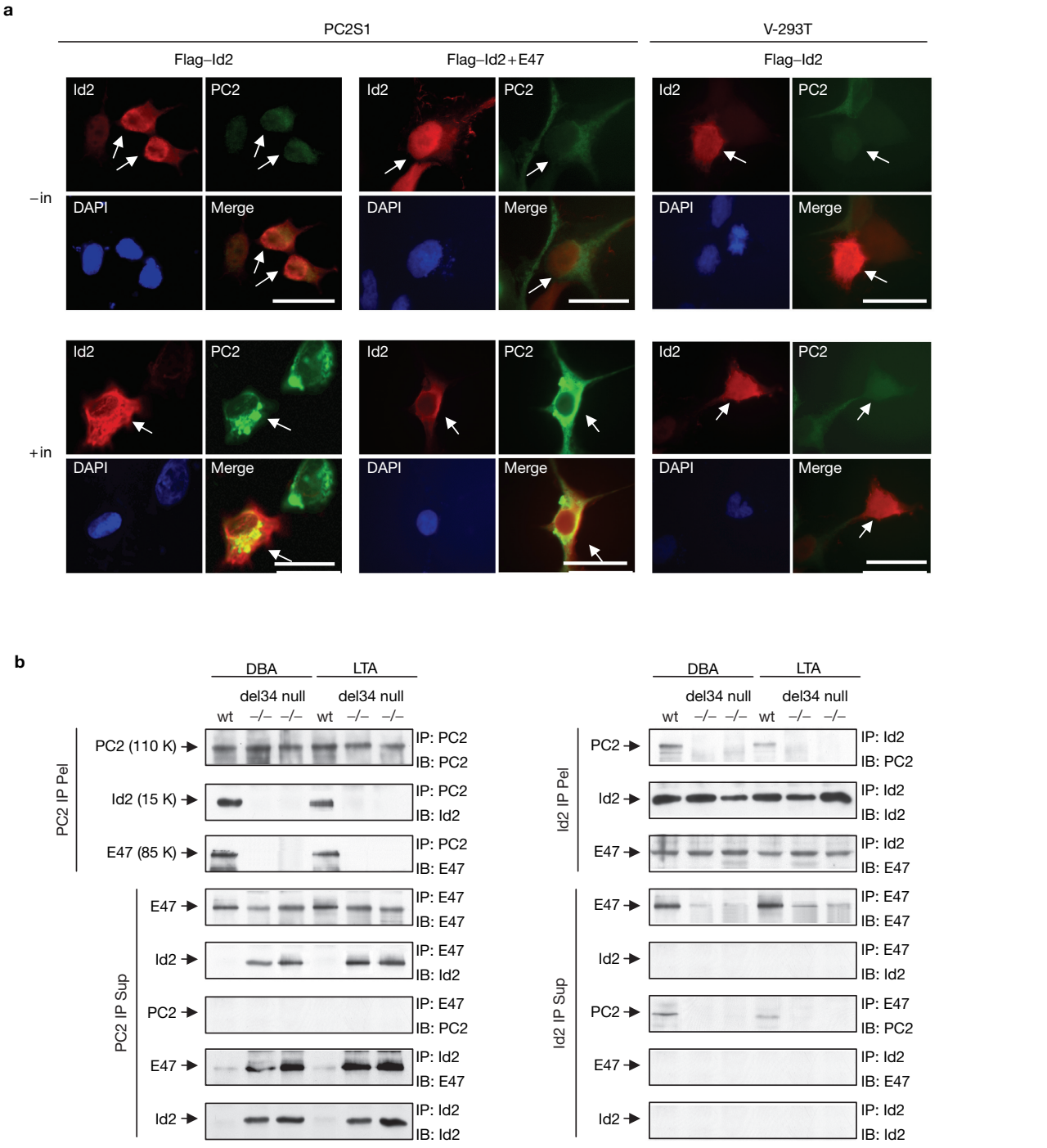

Figure 7 PC2 regulates the Id2–E47 protein complex. (**a**) Immunostaining of PC2S1 (left and middle panels) or V-293T cells (right panels) that have been transiently transfected with either Flag–Id2 (left panel) or Id2 and E47 (middle panel) under induced (bottom panel) or uninduced (top panel) conditions. Induction of PC2 sequestered Id2 in the cytosol of E47-transfected cells. Scale bars, 10 μm. (**b**) Analyses of both the pellet (Pel) and supernatant (Sup) of endogenous PC2 (left panels) or Id2 (right panels); immunoprecipitation (IP) in mouse epithelial kidney cell lysates with indicated antibodies. PC2 co-immunoprecipitated both Id2 and E47 in the wild-type but not in the PC1 mutant cells. E47, available in the supernatant of all six types of cell lysates, was bound to Id2 mostly in the PC1 mutant cells. Id2 co-precipitated PC2 and E47 in the wild-type cells, but only co-precipitated E47 in the mutant cells. IB, immunoblot; K, relative molecular mass in thousands.

and bone morphogenetic protein (BMP)-mediated pathways[44]. Sustained repression of the *Id2* gene was believed to be physiologically relevant to cell-cycle control. This is consistent with our finding that PC2 controls Id2 function by retaining it in the cytoplasm. Ectopic expression of Id2 attenuated growth inhibition by TGF-β1 and completely neutralized the weak BMP7 effects in epithelial cells[44].

In summary, we have demonstrated that Id2 is a downstream target for both PC1 and PC2, which play key regulatory roles in the control of epithelial-cell proliferation and differentiation. Our data also indicate that aberrant Id2 nuclear translocation resulting from loss-of-function mutations in either *PKD1* or *PKD2* contributes to abnormal cellular proliferation in ADPKD, which is a trigger for cyst formation.

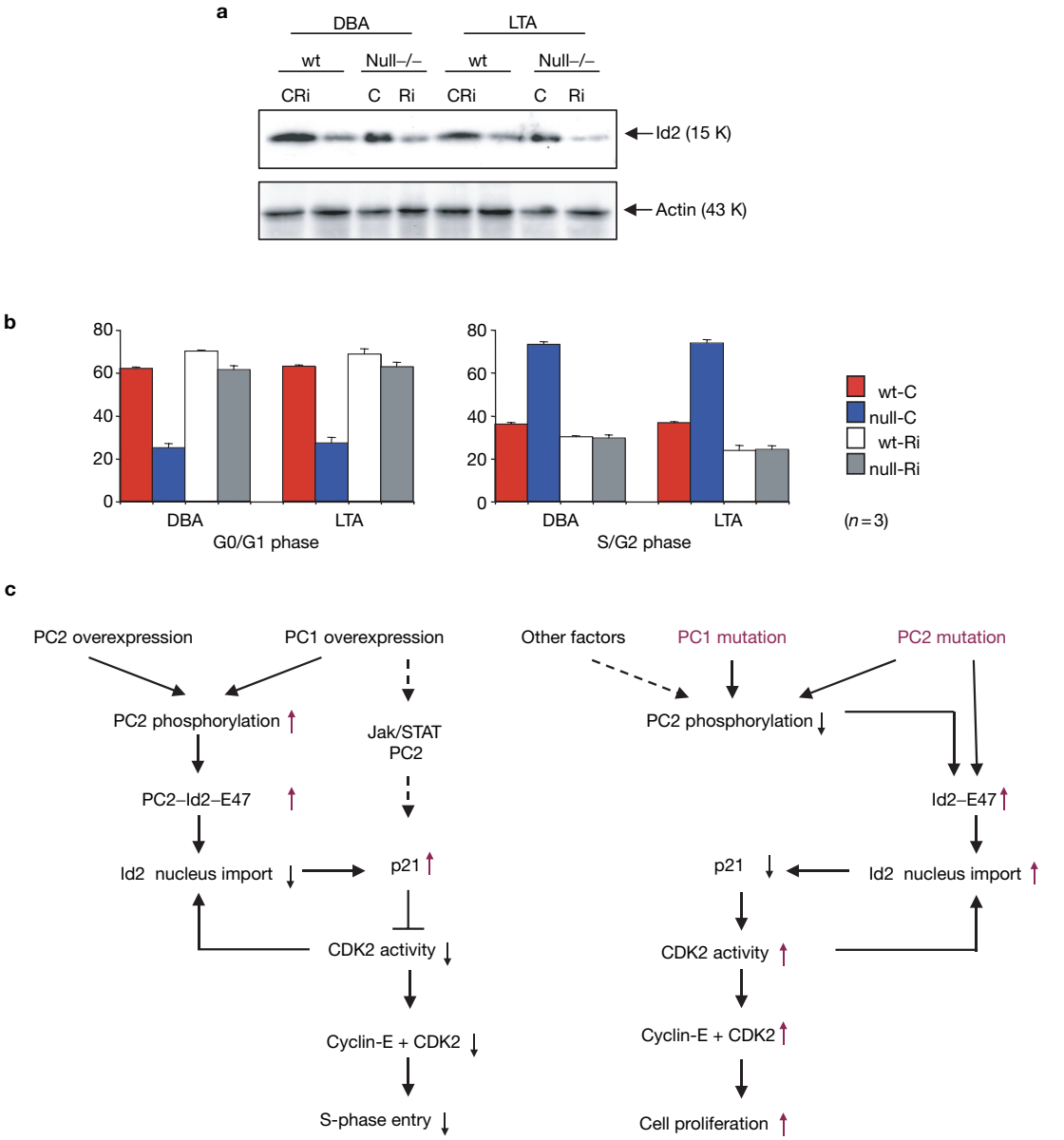

Figure 8 Id2RNAi and cell cycle. (**a**) Reduced Id2 expression by RNA interference (RNAi) shown by western blot analysis in 293T cells. A total of 50% of transfection efficiency of pBS/U6-EGFP/Id2 (Ri) correlated well with ~50% reduction of Id2 proteins. C, vector control. DBA, *dolichos biflorus agglutinin*; K, relative molecular mass in thousands; LTA, *lotus tetragonolobus*. (**b**) Id2 RNAi reversed the cell-cycle profile of the PC1 mutant cells to normal. Only green fluorescent protein (GFP)-positive cells were used for cell-cycle analyses. The histogram indicates the percentage of cells at G0/G1 and S/G2 phases with or without Id2 RNAi. Error bars indicate SEM. wt-C and null-C represent the wild type or *PKD1* null MEK cells transfected with the RNAi control vector. (**c**) Schematic diagram illustrates the potential mechanisms by which PC1 and PC2 regulate the cell cycle and cell proliferation through Id2. PC1 or PC2 expression induces PC2 phosphorylation and PC2–Id2 interactions, which retains the Id2–E47 complex in the cytosol and prevents Id2 repression of E-box-dependent activation of transcription, such as by p21, and controls cell proliferation. A previously identified PC1–STAT–p21 pathway is shown by the dashed line. Mutations in either PC1 or PC2 disrupt the PC2–Id2 interaction and cause abnormal cell proliferation.

A recent report supports our finding that the function of Id2 is regulated by nucleus–cytosol shuttling[45]. Restoration of the normal subcellular distribution of Id2 therefore presents a novel therapeutic strategy for the treatment of ADPKD. □

METHODS

Cell culture and cell-cycle analysis. Human embryonic kidney 293T (293T) cells and PC1 and PC2 stable overexpressing cells were maintained as previously described[20]. To determine cell proliferation rate, PC2 stable transfected (PC2S1) and parental cells were seeded in six-well plates with their medium being changed every 3 d. Triplicate cultures were counted at 24-h intervals by a cell counter. MEK cells isolated from E15.5 wild-type and *Pkd1* mutant mice (*Pkd1*$^{del34/del34}$ and *Pkd1*$^{null/null}$) that were positive for either DBA, a collecting duct marker, or LTA, a proximal tubule marker, were cultured at 33°C as described previously[21]. Cells were cultured to 30–50% confluency and transiently transfected with Fugene 6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. Cells were harvested for further analysis 48 h after transfection. For cell-cycle analysis, cells were synchronized by serum starvation for 24-h followed by addition of 10% serum for 24-h, stained with propidium iodide (PI) and analysd by FACS. For S-phase analysis with bromodeoxyuridine (BrdU) labelling, cells were transiently transfected with respective constructs, then pulsed with 20 μM BrdU (Roche) following the manufacturer's protocols. Fluorescein-conjugated anti-BrdU antibody (PharMingen, San Jose, CA; 1:100 dilution) was used for

FACS analyses. For each sample, 10,000 cells were scored using a FACScan flow cytometer. Cell-cycle distribution was analysed with Modfit software (Verity Software House, Inc., Topsham, ME).

Yeast two-hybrid screen. The entire coding C-terminal domain of human PC2 was subcloned into the bait plasmid *pGBK-T7* to create *pBD-c-PKD2*. AH109 yeast cells were transformed with the bait construct and a human fetal kidney cDNA library in the prey plasmid, pACT2, was screened according to the Matchmaker protocol (BD Clontech, Basingstoke, UK).

Luciferase assays. Cells transfected with luciferase reporter plasmids, p21-luc, and other constructs were plated in six-well plates. The p21-luc constructs contain a 2.3-kb fragment of the human *p21* promoter that was subcloned into the plasmid pGL3 (Promega Corp., Madison, WI) upstream of the luciferase reporter gene, using the *Xho*I and *Mlu*I sites on pGL3. Luciferase measurements were performed 24 h after transfection using a luminometer. Relative luciferase activity was normalized with cotransfected Renilla luciferase activity following the manufacturer's protocols (Promega Corp.).

Data analysis and statistics. All values for statistical significance represent mean $\pm$ SE. We carried out comparisons between means using the paired Student's *t*-test. All comparisons with negative results had statistical powers of ≥ 0.8, and statistical significance implies $P < 0.05$.

Immunoprecipitation and western blotting. Immunoprecipitation and western blotting were performed on whole-cell lysates (unless otherwise specified), as described by the manufacturer's (Upstate Biotechnology, Lake Placid, NY). Cytoplasmic and nuclear extraction was isolated as described by Pierce (Rockford, IL). The antibodies used for western blotting included rabbit polyclonal anti-Id2 (1:200, C-20; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Cdk2 (BD Pharmingen), rabbit polyclonal anti-p21 (H-164; Santa Cruz Biotechnology), rabbit polyclonal anti-phosphoserine (1:350; Chemicon International, Temecula, CA), rabbit anti-PC2 polyclonal antibody 96525 (ref. 20), rabbit anti-PC1 polyclonal antibody 96521 (ref. 21) and goat polyclonal anti-lamin B (M-20; Santa Cruz Biotechnology), rabbit polyclonal anti-α-tubulin (H-300; Santa Cruz Biotechnology), rabbit polyclonal anti-actin (H-196; Santa Cruz Biotechnology) and mouse monoclonal anti-Flag M2 antibody (Sigma, St Louis, MO).

All primary antibodies were used at 1:50 for immunoprecipitation and 1:500 for western blotting, unless specified. Goat-anti-rabbit Ig-horseradish peroxidase (HRP or goat-anti-mouse IgG–HRP, 1:10,000 dilution; Amersham Pharmacia Biotech, Piscataway, NJ) was used as a secondary antibody for western blotting.

Immunocytochemistry and immunohistochemistry. Immunofluorescence was carried out as described previously[20]. Primary antibodies were used at the following dilutions: Id2 (1:50), PC1 (1:500), PC2 (1:500) and Flag (1:500). The Id2 histology data obtained with the Santa Cruz antibody was confirmed by using another anti-Id2 antibody (1:50; 18-7398; Zymed, San Francisco, CA) on Id2 wild-type and knockout kidney tissues (see Supplementary Information, Fig. S1), as well as normal human and patient kidney tissues (Fig. 4a). Secondary antibodies used included goat-anti-rabbit IgG-fluorescein isothiocyanate (Molecular Probes, Eugene, OR) and goat-anti-mouse IgG-Texas Red (1:500 dilution; Molecular Probes). Confocal microscope 1024 MRC (Kr/Ar laser) (Bio-Rad, Hercules, CA) was used to acquire images for human and mouse tissues. The same exposure was used for the same antibody.

For paraffin-embedded sections, we used a method that was modified from that recommended by Upstate. Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized using xylene and were rehydrated using a graded series of alcohol solutions, followed by antigen retrieval in 10 mM sodium citrate, pH 6.0. Sections were blocked by normal goat serum (DAKO, Carpinteria, CA) for 1 h, followed by overnight blocking with 5% bovine serum albumin/phosphate-buffered saline. Primary and secondary antibodies were then applied as described above.

Inhibition of Id2 or PC2 by siRNAs synthesized from DNA templates *in vivo*. Id2 and PC2 RNAi vectors were made using pBS/U6-EGFP (gift from Y. Shi, Harvard Medical School, Boston, MA), which includes an enhamced GFP gene as a marker for transfection as described (Ambion, Austin, TX). Plasmid pBS/U6-EGFP was digested with *Apa*I and *Eco*RI. Primer sequences are available on request. RNAi constructs were transiently transfected into respective cell lines. The siRNA effect on the cell cycle of MEK cells or HEK293 cells was investigated by sorting the GFP-positive cells and by FACS analysis.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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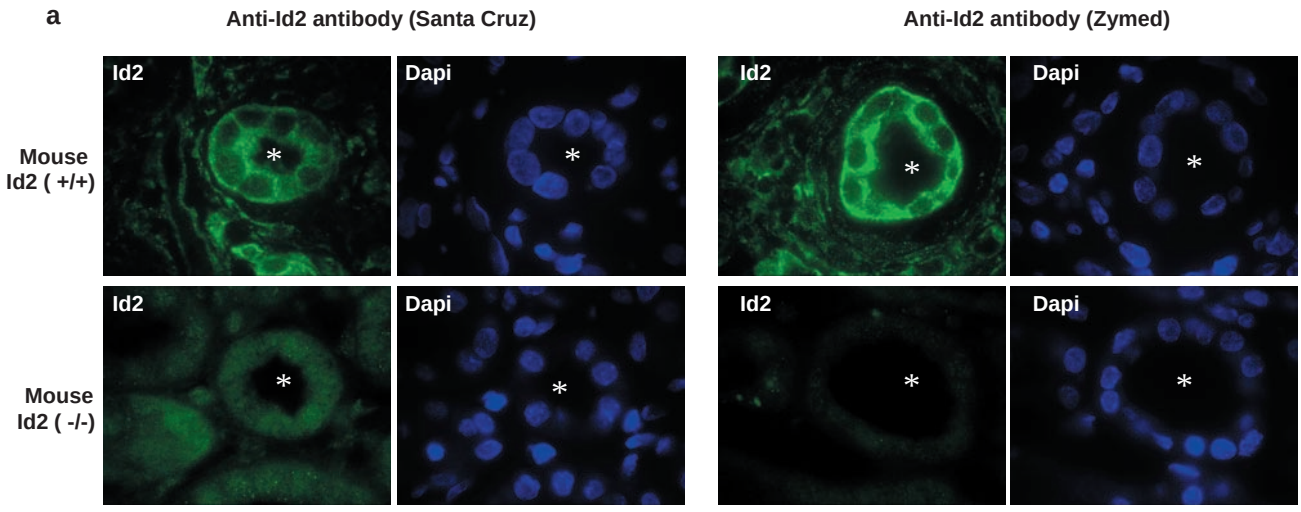

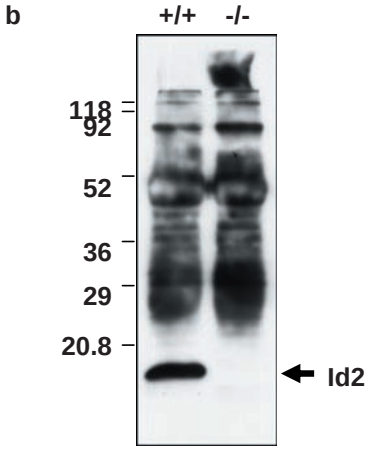

Supplemental Figure 1: a, Comparison of the Id2 antibody from Santa Cruz (left panels) and from Zymed (right panels). Id2 can be detected in the wild-type mouse kidneys with either Id2 antibody. In the Id2 knock-out kidneys, neither antibody detected any specific signals by immunofluorescence. Original magnification, 100 X. *, indicates lumen. Id2 images were captured with the same exposure time. b, Immunoprecipitation of Id2 with an antibody from Santa Cruz in wild-type and Id2-/- kidneys.