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Identification of an N-terminal glycogen synthase kinase 3 phosphorylation site which regulates the functional localisation of polycystin-2 *in vivo* and *in vitro*

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Abstract

PKD2 is mutated in 15% of patients with autosomal dominant polycystic kidney disease (ADPKD). Polycystin-2 (PC2), the PKD2 protein, is a nonselective Ca^{2+}-permeable cation channel which may function at the cell surface and ER. Nevertheless, the factors that regulate the dynamic translocation of PC2 between the ER and other compartments are not well understood. Constitutive phosphorylation of PC2 at a single C-terminal site (Ser^{812}) has been previously reported. Since we were unable to abolish phospholabelling of PC2 in HEK293 cells by site-directed mutagenesis of Ser^{812} or all 5 predicted phosphorylation sites in the C-terminus, we hypothesised that PC2 could also be phosphorylated at the N-terminus. In this paper, we report the identification of a new phosphorylation site for PC2 within its N-terminal domain (Ser^{76}) and demonstrate that this residue is phosphorylated by glycogen synthase kinase 3 (GSK-3). The consensus recognition sequence for GSK-3 (Ser^{76}/Ser^{80}) is evolutionarily conserved down to lower vertebrates. In the presence of specific GSK-3 inhibitors, the lateral plasma membrane pool of endogenous PC2 redistributes into an intracellular compartment in MDCK cells without a change in primary cilia localization. Finally, co-injection of wild-type but not a S76A/S80A mutant *PKD2* capped mRNA could rescue the cystic phenotype induced by an antisense morpholino oligonucleotide to *pkd2* in zebrafish pronephric kidney. We conclude that surface localization of PC2 is regulated by phosphorylation at a unique GSK-3 site in its N-terminal domain *in vivo* and *in vitro.* This site is functionally significant for the maintenance of normal glomerular and tubular morphology.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited human renal disease (incidence 1 in 1000 live births) and is caused by mutations in two genes, *PKD1* (85%) and *PKD2* (15%). (1)ADPKD is an important cause of end-stage renal failure, accounting for ~10% of patients on renal replacement therapy. Typically, fluid-filled cysts form in the kidney but cysts also commonly arise in the liver and pancreas. There is also an increased incidence of non-cystic extrarenal manifestations in ADPKD such as cardiac valve abnormalities, diverticular disease and intracranial aneurysms. (2)

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The ADPKD proteins, polycystin-1 (PC1) and polycystin-2 (PC2) are believed to function as a complex, activating a number of key signalling pathways which in turn regulate diverse cellular functions including proliferation, apoptosis, tubulogenesis and fluid secretion. (3)This is consistent with the largely overlapping renal and extrarenal phenotypes of PKD1 and PKD2 patients. (3)PC1 may play a role in mediating cell adhesion or mechanosensation. (4,5)PC2 has been shown to function as a nonselective Ca^{2+}-permeable cation channel, forming part of the TRP (transient receptor potential) superfamily of channels which broadly function as cellular sensors for multiple stimuli. (6,7)Although both proteins probably function together as a heterodimeric complex, more recent work suggests that they can also function independently of the other. (8,9)

Although PC1 is believed to act at the plasma membrane, the major site of action of PC2 has been debated. Endogenous PC2 channel activity has been detected in the apical membrane of placental syncytiotrophoblasts, primary cilia and the plasma membrane of cultured kidney cells such as mIMCD3 and LLCPK. (10-13)Conversely, heterologous PC2 has been shown to function as a calcium-activated ER calcium channel in LLCPK cells. (14)These findings could be reconciled if PC2 is active at more than one subcellular location (e.g. ER, primary cilia, plasma membrane).

Protein phosphorylation is an important post-translational mechanism known to regulate the function of many proteins by affecting their assembly, retention, targeting, retrieval, activity or half-life. (15)An effective means to control the activity of PC2 at the cell surface would be to regulate its targeting, trafficking or retention between the ER, the primary cilia or lateral plasma membrane. Recent work has suggested that PC2 is phosphorylated at residue Ser^{812} in its C-terminus and that this event is important for its recognition and retrieval from the plasma membrane to the Golgi and ER. (16,17)In this paper, we report the identification of an alternative phosphorylation site within the N-terminus of PC2, and demonstrate that it is critical for the function of PC2 at the plasma membrane both *in vitro* and *in vivo*.

Results

PC2 is phosphorylated at both its N- and C-termini

Sequence analysis of human PC2 by prediction programs including PhosphoBase, DISPHOS 1.3 and NetPhos show that it contains a large number of predicted phosphorylation sites within both its N and C-terminal cytoplasmic domains. In agreement with a previous study, we were able to demonstrate that PC2 is constitutively phosphorylated *in vivo*. (16)Endogenous PC2 in mouse collecting duct cells was metabolically labelled with ^{32}Pi - autoradiography of the immunoprecipitated protein revealed a single band migrating at 110 kDa by SDS-PAGE which was absent in the control lane (Fig. 1A). Immunoblotting of the same membrane with p30 confirmed that the phosphorylated protein was indeed PC2 (data not shown).

To determine the specific residues phosphorylated in PC2, we substituted alanine or glycine at various combinations of five predicted phosphorylation sites within the C terminus, Thr^{721}, Ser^{801}, Ser^{812}, Ser^{831} and Ser^{943}. (18)In addition, a naturally occurring truncation mutant, R742X, which lacks most of the PC2 C-terminus but which does contain the Thr^{721} site was generated (Fig. 1B). *In vivo* phosphorylation analysis was performed by metabolic labelling of transiently transfected HEK-293 cells (Fig. 1C). However, unlike a previous study, we were unable to eliminate phosphorylation of PC2 by mutation of Ser^{812} (Fig. 1C) Moreover, mutation of all 5 predicted sites within the C-terminus did not abolish phospholabelling though a reduced level of phosphorylation was detected (Fig. 1C). R742X-PC2 also showed a reduced level of phospholabelling (Fig. 1C). Although this contains the Thr^{721} residue, mutation of Thr^{721} alone did not significantly alter PC2 phosphorylation, consistent with previous findings. (16)

These findings led us to hypothesize that PC2 could be phosphorylated within its N-terminal domain. In the original report describing the cloning of *PKD2*, an N-terminal residue, Ser^{122}, was predicted to be a putative site for phosphorylation. (18)To investigate this further, the entire N-terminal cytoplamic domain of PC2 (aa1-224) was first generated with an N-terminal HA epitope tag for detection. Two additional constructs containing progressive truncations of the PC2 N-terminus (aa1-178, aa1-118) were subsequently generated for further study (Fig. 1D). When L224X was transiently expressed in HEK-293 cells, a doublet band was unexpectedly observed on western blotting using an HA epitope specific antibody (Fig. 1E, upper panel). We speculated that the upper band represented a phosphorylated form of the protein. Dephosphorylation with the enzyme, lambda phosphatase (which removes all phosphate groups from serine, threonine or tyrosine residues) resulted in loss of the upper band in a time dependent manner, confirming that the reduction in mobility on SDS-PAGE was indeed the result of phosphorylation (Fig. 1E, upper panel). *In vivo* phospholabelling further demonstrated that L224X could indeed be phosphorylated in HEK-293 cells (Fig. 1F).

Despite the prediction that an N-terminal residue, Ser^{122}, might be the major site of phosphorylation in the N-terminus, we observed phospholabelling of the smallest N-terminal construct, W118X, which contains the first 118 amino acids of PC2, in HEK-293 cells (Fig. 1F). Once again, a doublet band was observed on immunoblotting which could be abolished by lambda phosphatase treatment (Fig. 1E, lower panel). From these results, we concluded that the major site of PC2 phosphorylation within its N-terminal cytoplasmic domain might lie between amino acids 1 and 118.

GSK-3 mediates phosphorylation of the N-terminal of PC2 at Ser^{76}

Using prediction programmes, a number of phosphorylation sites (Ser^{74}, Ser^{76}, Ser^{122}, Ser^{125}) can be predicted within the N-terminus of PC2 though none have been previously studied. To determine which of these sites might be phosphorylated (Fig 1E and F), we mutated each of the residues shown in Fig 2A in L224X and analysed each mutant in turn as described above. Mutation of Ser^{74}, Ser^{122} or Ser^{125} did not affect phosphorylation of L224X as assessed by doublet band formation (Fig 2B upper panel) or labelling with ^{32}P (Fig 2B lower panel). However, mutation of Ser^{76} to Ala^{76} resulted in a complete loss of labelling indicating it is the major residue phosphorylated within the N-terminus of PC2 (Fig 2B).

Apart from HEK-293 cells, the PC2 N-terminal domain could be phosphorylated in a number of cell lines including IMCD3, CHO-K1 and MDCK as assessed by doublet band formation on immunoblotting (Fig. 2C). This phosphorylation was absent in cells transfected with a Ser^{76} mutant construct (Fig. 2C). Ser^{76} in human PC2 is highly conserved in PC2 homologues from higher and lower vertebrates suggesting that it has retained an important functional significance throughout evolution (Fig. 2D). It forms part of a GSK-3 recognition sequence, Ser/Thr-X-X-X-Ser/Thr, which is also fully conserved. This recognition sequence was however not conserved in fly, worm or sea urchin PC2 homologues suggesting that the function of PC2 is different in these lower organisms (data not shown).

To determine whether GSK-3 could directly phosphorylate the PC2 N-terminus, we expressed L224X in HEK-293 cells in the presence or absence of four specific GSK-3 inhibitors (LiCl, (2′*Z*,3′*E*)-6-Bromoindirubin-3′-oxime, SB 415286 and SB 216763). In each case, incubation with the inhibitor resulted in the loss of the phosphorylated upper band (Fig. 2E).

Substrates of GSK-3 typically require a priming phosphorylation event at a serine or threonine residue four bases downstream of the GSK-3 phosphorylation site (Fig. 2D). To determine whether mutation of Ser^{80} had any effect on phosphorylation of the PC2 N-terminus, we mutated it to an alanine and examined the effect on phosphorylation of L224X. As shown in Fig 2F, the phosphorylated upper band was significantly reduced following mutation of

Ser^{80}. This suggests that Ser^{80} may be a priming phosphorylation site, which when absent, significantly reduces the ability of GSK-3 to phosphorylate Ser^{76}.

Inhibition of GSK-3 mediated PC2 phosphorylation alters its cellular localisation

To investigate the functional significance of GSK-3 phosphorylation of PC2 at Ser^{76}, the effect of GSK-3 inhibition on the subcellular localisation of endogenous PC2 was examined in the polarised kidney cell line, MDCK. Cells were treated with either LiCl or SB 415286 for 16 hours and the distribution of endogenous PC2 and other well characterised adhesion junction proteins examined by immunofluorescence (Fig 3A). In the presence of specific GSK-3 inhibitors, the lateral membrane pool of PC2 redistributed into an intracellular compartment in MDCK cells with no change in its localisation in primary cilia (Fig 3A). In contrast, the surface distribution of E-cadherin, ZO-1 and desmoplakin were unaffected after GSK-3 inhibition (Fig 3A). Similar results were seen in mIMCD3 cells and the mouse collecting duct cell line, M8, though the surface membrane signals were variable between lines (not shown). These results suggest that phosphorylation of PC2 by GSK-3 can affect its plasma membrane localisation independent of its ciliary location.

Redistribution of PC2 from the cell surface into an intracellular compartment following GSK-3 inhibition was confirmed by cell surface biotinylation in mIMCD3 cells which express the highest levels of PC2 (Fig 3B). mIMCD3 cells treated with SB 415286 showed a significant reduction in expression of PC2 at the cell surface following GSK-3 inhibition (Fig 3B). By densitometry, approximately 10% of total PC2 was present at the cell surface in control cells compared with ~ 1.5% after treatment with the inhibitor in 3 independent experiments. Similar results were obtained in MDCK cells (Fig. 3B). Biotinylation of cell surface proteins was shown to be specific as the ER resident protein calnexin was not biotinylated on reprobing of the membrane (Fig 3B). Na^+K^+ATPase, a cell surface protein was strongly biotinylated but was unaffected by treatment with the inhibitor (Fig 3B). These results confirm previous reports that a small plasma membrane population of PC2 can be surface biotinylated in mIMCD3 and MDCK cells. (12,19)

The phenotype induced by a *pkd2* antisense morpholino oligonucleotide (*pkd2*ATGMO) in zebrafish embryos can be rescued by wild type human *PKD2* but not a *PKD2* -S76A/S80A mutant mRNA

Since the GSK-3 consensus sequence was conserved in zebrafish *pkd2* (Fig. 2D), we reasoned that phosphorylation at this site might be functionally important for nephrogenesis in this organism. A *pkd2*ATGMO which has been shown to induce a cystic phenotype in zebrafish embryos by blocking zebrafish *pkd2* translation has been recently reported (20). Following injection of *pkd2*ATGMO into embryos, *pkd2* mRNA was not detected at 24 hpf (hours post fertilisation) demonstrating that this MO effectively induces a null phenotype (data not shown).

Similar to a previous report (20), we found that injection of *pkd2*ATGMO into zebrafish embryos (n=920) induced a characteristic phenotype at 72 hpf consisting of pronephric kidney cysts, body axis curvature and hydrocephalus in 880 embryos (95.6%, Fig 4B). Cystic dilatation of the pronephric tubules and glomeruli can be clearly seen in cross-section (Fig 4B). The specificity of this null phenotype was confirmed by its complete rescue by the over-expression of zebrafish *pkd2* cDNA (data not shown), the lack of effect in 815 control MO injected embryos (Fig 4A) and by the near-complete suppression of the *pkd2*ATGMO phenotype when 50pg human *PKD2* mRNA was co-injected - 783 out of 812 embryos rescued (96.4%, Fig 4C). In contrast, co-injection of 50pg human *PKD2*-S76A/S80A mutant mRNA could not rescue the pronephric cysts and hydrocephalus caused by *pkd2*ATGMO although body axis curvature was slightly rescued in the majority – 761 out of 792 embryos (96.1%, Fig 4D). The remaining

31 embryos (3.9%) showed the same phenotype as with *pkd2*ATGMO indicating a complete lack of rescue.

RT-PCR analysis using primers for the N-terminus of *PKD2* confirmed expression of both wild-type *PKD2* and *PKD2*-S76A/S80A mRNA in the respective co-injected embryos (Fig 4E). PCR products were sequenced to confirm the absence or presence of the mutated residues (data not shown).

Discussion

The subcellular localisation of PC2 has been the subject of some debate due to different findings in different experimental systems. In summary, PC2 has been localised to the ER, plasma membrane (basolateral or apical) and primary cilia: functional channel activity at all these sites has been documented. For instance, endogenous surface PC2 currents have been measured in human syncytiotrophoblasts, mIMCD3 and LLCPK1 cells. (10,12,13)In addition, flow-activated Ca^{2+} currents attributable to ciliary PC2 channels are present in immortalised mouse collecting duct cells. (11)Similarly, ER microsomes isolated from LLCPK1 stably transfected with heterologous *PKD2* demonstrate PC2 currents. (14)Differences in cell type, differentiation status and culture conditions may account for some of these differences. (12, 21)Nevertheless, it is unclear whether the loss of PC2 function within a specific compartment is especially critical for the maintenance of tubular differentiation and morphology.

Subcellular membrane fractionation has shown that most of native PC2 (~90%) is located in the ER with smaller amounts in the Golgi and plasma membrane. (8,21)The plasma membrane component (including ciliary membrane) can vary but probably accounts for ~5-10% of total cellular PC2, as indicated by previous reports and from our current study. This has been demonstrated using a variety of techniques such as membrane fractionation, surface biotinylation and live cell surface antibody labelling. (8,12,13,21)The surface fraction of PC2 is reported to be increased by the use of proteosome inhibitors and/or chemical chaperones in some experimental systems. (22)However it is variable and can be difficult to detect by immunolabelling. Nonetheless, it could represent the active population of PC2 channels, either as homodimers or as heterodimers with PC1 or other membrane proteins such as TRP channel subunits. (7,8)It is tempting to speculate that the targeting, anterograde or retrograde trafficking of PC2, from the ER to the plasma membrane or primary cilium are significant rate-limiting steps. The situation may be analogous to that of the epithelial sodium channel ENaC which is also found mainly as an ER resident and yet exerts its major function at the plasma membrane. (23)Similarly, the surface expression and activity of the TRP channel, TRPC5, can be induced by growth factors. (24)This could be an alternative mechanism for regulating TRP channel activity since many TRP channels are constitutively active. (25)

In a recent study, the retrograde trafficking of PC2 was shown to be dependent on phosphorylation at a single C-terminal residue, Ser^{812}, within an acidic cluster motif (SEED). (17)Phosphorylation at this casein kinase 2 (CK2) site enabled PC2 to be recognised by phosphofurin acidic cluster sorting protein-1 and -2 (PACS-1 and PACS-2), two adaptor proteins which in turn mediate the retrograde trafficking of PC2 from the plasma membrane to the Golgi, and Golgi to ER respectively.

Only one previous study has examined the phosphorylation of PC2, concluding that a single residue (Ser^{812}) within its C-terminal domain could be metabolically labelled *in vitro*. (16) Nonetheless, our results indicate that phosphorylation of PC2 can also occur within the N-terminal domain at a different residue (Ser^{76}) which forms part of a highly conserved GSK-3 recognition consensus sequence. Its functional importance was further demonstrated by the loss of surface PC2 in MDCK and mIMCD-3 cells in the presence of chemical GSK-3

inhibitors. Significantly, we found that the phenotype induced by a *pkd2*ATGMO to zebrafish (cystic pronephros, body curvature, and hydrocephalus) was completely rescued by capped wild-type but not by a S76A/S80A *PKD2* mutant mRNA indicating that GSK-3 modification of PC2 is functionally important for the maintenance of normal glomerular and tubular morphology (Fig 4).

It seems likely that there exists a different targeting ciliary motif for PC2 since GSK-3 inhibition had no effect on PC2 localisation in primary cilia (Fig 3A). Whatever the significance of ciliary PC2 in zebrafish pronephric kidney, our results suggest that the plasma membrane localisation of PC2 is itself functionally important. The dynamic control of PC2 insertion into the plasma membrane by phosphorylation at two specific residues (Ser^{76} or Ser^{812}) provides further evidence of how tightly the activity of this channel is regulated (Fig 3C). Finally, mutation of both Ser^{76} and Ser^{812} residues was insufficient to completely abolish phospholabelling of PC2 (data not shown). Characterisation of other phosphorylation sites within PC2 will be the subject of further study.

Materials and methods

Materials

All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. MDCK II cells were a gift of Prof R Montesano (Geneva, Switzerland) and HEK-293 cells were provided by Dr S Ponnambalam (Leeds, UK). A mouse mAb to desmoplakin (clone 115F) was provided by Professor DR Garrod (Manchester, UK). A rat mAb to ZO-1 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA. Antibodies to E-cadherin and calnexin were purchased from BD Transduction Labs (Cowley, Oxford, UK). The PC2 antibodies p30 and 1A11 (gift of Dr G Wu, Vanderbilt, USA) which recognise the C-terminal 258 amino acids of human PC2 have been previously described. (26,27)

Cell Culture and Transfection

HEK-293, MDCK and mIMCD3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transient transfection was carried out on cells cultured to 90% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Conditionally immortalised mouse collecting duct cells (M8) were cultured at 33°C in DMEM/ F12 supplemented with 5% Nuserum and recombinant mouse gamma-interferon (Boehringer Mannheim, Germany) to activate T antigen expression as previously described. (8)

^{32}P Metabolic Labelling

Cells were cultured to 90% confluency prior to labelling. Cells were washed 3x phosphate deficient DMEM to remove any residual phosphate and incubated for 4 h in phosphate deficient medium containing inorganic ^{32}P at 50 μCi/ml. The radio-labelling was terminated by washing the cells twice in with ice-cold PBS. For immuno-precipitation (IP), cells were lysed in IP buffer (150 mM NaCl, 25 mM $NaPO_4$ pH 6.9, 1% TX100, 0.5% NP40 containing 50 mM sodium fluoride, 2 mM sodium orthovanadate and complete protease inhibitors) for 1h at 4°C on a rotator or with 1% SDS. (8) Lysates obtained with 1% SDS were diluted ten-fold with IP buffer (final SDS concentration 0.1% SDS) prior to IP. The supernatants recovered by centrifugation were then immuno-precipitated using the antibodies described and immunocomplexes resolved on SDS-PAGE. The gels were dried and subjected to auto-radiography.

Lambda Protein Phosphatase Assay

Following transfection cells were cultured for 48 h and lysed in IP buffer containing complete protease inhibitors for 1 h at 4°C on a rotator. Dephosphorylation was carried out by incubating 50 μg total cell lysates with 200 U Lambda (λ) protein phosphatase (New England Biolabs) for 30, 60 and 90 min at 30°C in reaction buffer (50mM Tris-HCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35 pH 7.5) supplemented with 2 mM $MnCl_2$. Reactions were terminated by addition of an equal volume of 2x sample loading buffer. Lysates were analysed by SDS-PAGE and western blotting as previously described. (8)

Site Directed Mutagenesis

Site directed mutagenesis was carried out using the Quickchange site-directed mutagenesis kit (Stratagene, UK) according to the manufacturers instructions. The full-length *PKD2* plasmids, PKD2Pk and TM4FL (gift of Prof S Somlo, Yale, USA) have been described and were used as templates. (18,26)PAGE purified mutagenic primers were synthesised by Sigma Genosys (UK) - primer sequences are available on request.

Generation of PC2 truncation mutants

We introduced the naturally occurring truncating *PKD2* mutation R742X by polymerase chain reaction (PCR) using the PKD2Pk plasmid as a template. An influenza virus hemagglutinin (HA) protein epitope tag and an in-frame stop codon were included in the reverse primer. *PKD2* cDNAs containing codons 1-224 (full N-terminus) and truncations 1-178 and 1-118 were generated by PCR amplification from the TM4FL template using forward and reverse primers containing *Xba*I and *Hind*III linkers respectively. The forward primer containing the *Xba*I site also contained an HA tag sequence. PCR products were gel purified and subcloned into the *Xba*I and *Hind*III sites of the expression plasmid pcDNA3.1 (−). All cDNA constructs were confirmed by direct sequencing. PCR primer sequences are available on request.

Cell Surface Biotinylation

Cells were cultured to confluency in 10cm dishes, washed three times with PBS and incubated for 30 min with 1 mg/ml sulpho-NHS-LC-Biotin in PBS at 4°C. Cells were rinsed twice with PBS containing 100 mM glycine and excess biotin quenched by further incubation for 20 min with PBS containing 100 mM glycine and 0.1% BSA. Cells were rinsed twice with PBS and lysed by the addition of 500 μl lysis buffer (50 mM Tris, 500 mM NaCl, 5mM EDTA, 1% Tx100, pH 7.5) for 1 h at 4°C. Equal amounts of cell lysate were incubated with 100 μl streptavidin beads overnight at 4°C. Beads were washed three times each with lysis buffer, high salt buffer (50 mM Tris, 500 mM NaCl, 0.1% Tx100) and non-salt buffer (10 mM Tris, pH 7.5) before being resuspended in 40 μl 2 × SDS sample buffer. Samples were analysed by SDS-PAGE and western blotting using the antibodies described.

Immunofluorescence

Cells were grown on glass coverslips and were fixed with ice-cold methanol:acetone (70:30) at 4°C for 10 min followed by air drying for 10 min. Blocking was carried out for 15 min with 5% BSA/PBS and primary antibodies were incubated overnight at 4°C in 1% BSA/PBS. Controls included cells stained with primary antibody omitted, an irrelevant IgG1 mAb (Serotec, Kidlington, UK) or a non-immune rabbit IgG fraction (Dako, Ely, UK). Antibody binding was visualised using FITC-conjugated goat ant-mouse IgG and Texas Red or FITC labelled goat anti-rabbit secondary antibodies. Slides were viewed using an Olympus Imaging Systems inverted IX71 microscope configured for multi-fluorescence image capture. Images were acquired and analysed using SimplePCI imaging software (Compix, Inc).

Zebrafish experiments

Wild-type zebrafish AB strain were maintained and raised as described. (28)Embryos were kept in E3 solution and staged according to hours post-fertilization (hpf). (29) An antisense morpholino oligonucleotide (MO, Gene Tools LLC Philomath, Oregon, USA) was used to block zebrafish *pkd2* translation as described (hi4166). (20) Final *pkd2*ATGMO concentration in the cytoplasm was estimated as 100 nM. The rescue experiments were carried out by co-injecting 50 pg of *in vitro* transcribed capped mRNA encoding human *PKD2*-wt or *PKD2*-S76A/S80A. Capped RNAs were synthesized using a mMessage mMachine T7 kit (Ambion, Austin, Texas, USA). Embryos were microinjected at the one cell stage in the yolk in 100mM KCl, 0.1% Phenol Red and 10mM HEPES. Nested RT-PCR primers were used to detect human *PKD2*-wt or *PKD 2*-S76A/S80A to confirm expression in the injected zebrafish embryos. Amplification of *β-actin* was performed as a positive control. (30)Primers designed to detect human *PKD2* are *hPKD2*-13F1: AGTCGCGTGCAGCCTCAG, *hPKD2*-358R1: GGCGCCACTCTACGTCCAT, *hPKD2*-152F2: GCCTGGAGATCGAGATGC, *hPKD2*-323R2: CCTTCTTCCCCTTCCACCT. Embryos were fixed at 72hpf in 1.5% Glutaraldehyde, 1% Paraformaldehyde, 70mM NaPO4 pH 7.2, 3% Sucrose overnight at 4°C, embedded in glycolmethacrylate (JB-4; Polyscience, Warrington, Pennsylvania, USA) by following the manufacturer's instructions and sectioned at 4μm using HM325 (Richard-Allan Scientific). Slides were stained with Methylene Blue and Azure II. (31)Whole mount images are taken by Leica MZ125 and section images are taken by Nikon E500 and analyzed by Spot v. 4.2 program.

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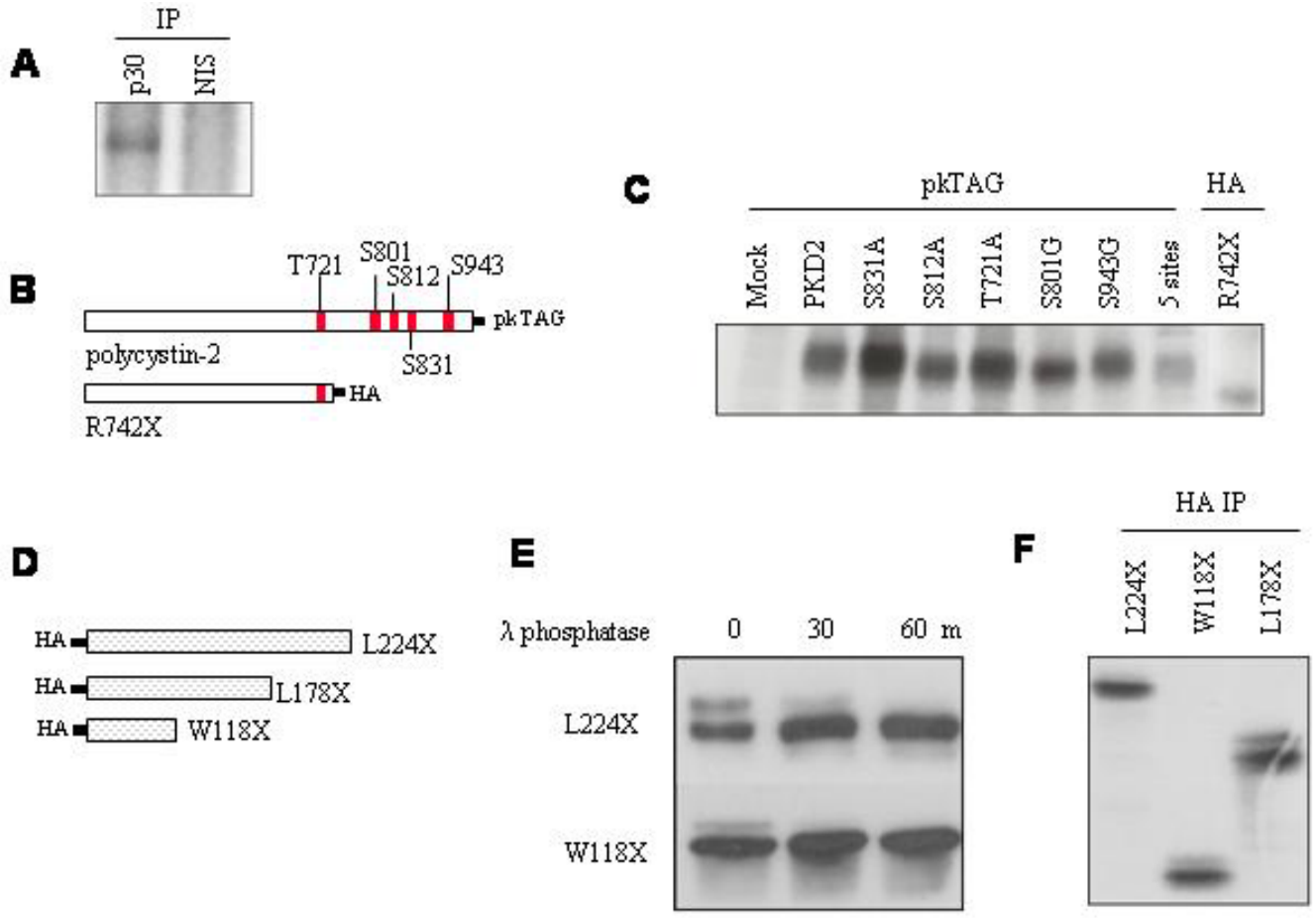

Fig 1. PC2 is phosphorylated at both its N- and C-termini

(A). Endogenous PC2 was shown to be phosphorylated by immuno-precipitation of ^{32}P labelled samples derived from mouse collecting duct cells. The rabbit polyclonal antibody p30 and non-immune rabbit serum (NIS) control was used for IP.

(B). A diagram of PC2 full length and R742X truncated constructs showing the predicted sites of phosphorylation within the C-terminus which were mutated for phosphorylation analysis.

(C). Immunoprecipitation of PC2 constructs from transfected HEK-293 cell lysates was carried out following metabolic labelling with ^{32}P. Labelled constructs were immunoprecipitated with antibodies raised to a pkTAG epitope tag or antibodies raised to an HA epitope tag (R742X). All C-terminal mutants as well as wild type PC2 were shown to be phosphorylated. However the 5 site combined mutant showed a reduced level of phosphorylation compared to the single site mutants. The R742X construct was also labelled with ^{32}P.

(D). A diagram showing the constructs generated to assess phosphorylation of the N-terminus of PC2.

(E). Cell lysates from HEK-293 cells were treated with λ phosphatase for 0, 30 and 60 min and analysed by western blotting with HA tag specific antibodies. A clear doublet band is seen in both L224X transfected cells (upper panel) and W118X transfected cells (lower panel). The upper band disappears following incubation with the enzyme demonstrating that it is due to phosphorylation.

(F). Immunoprecipitation of PC2 N-terminal constructs truncated at amino acids 118, 178 and 224 was carried out following metabolic labelling with ^{32}P in transfected HEK-293 cells. Labelled constructs were immunoprecipitated with antibodies raised to an HA epitope tag,

separated on 5% SDS-PAGE and analysed by autoradiography. All three constructs were shown to be phosphorylated.

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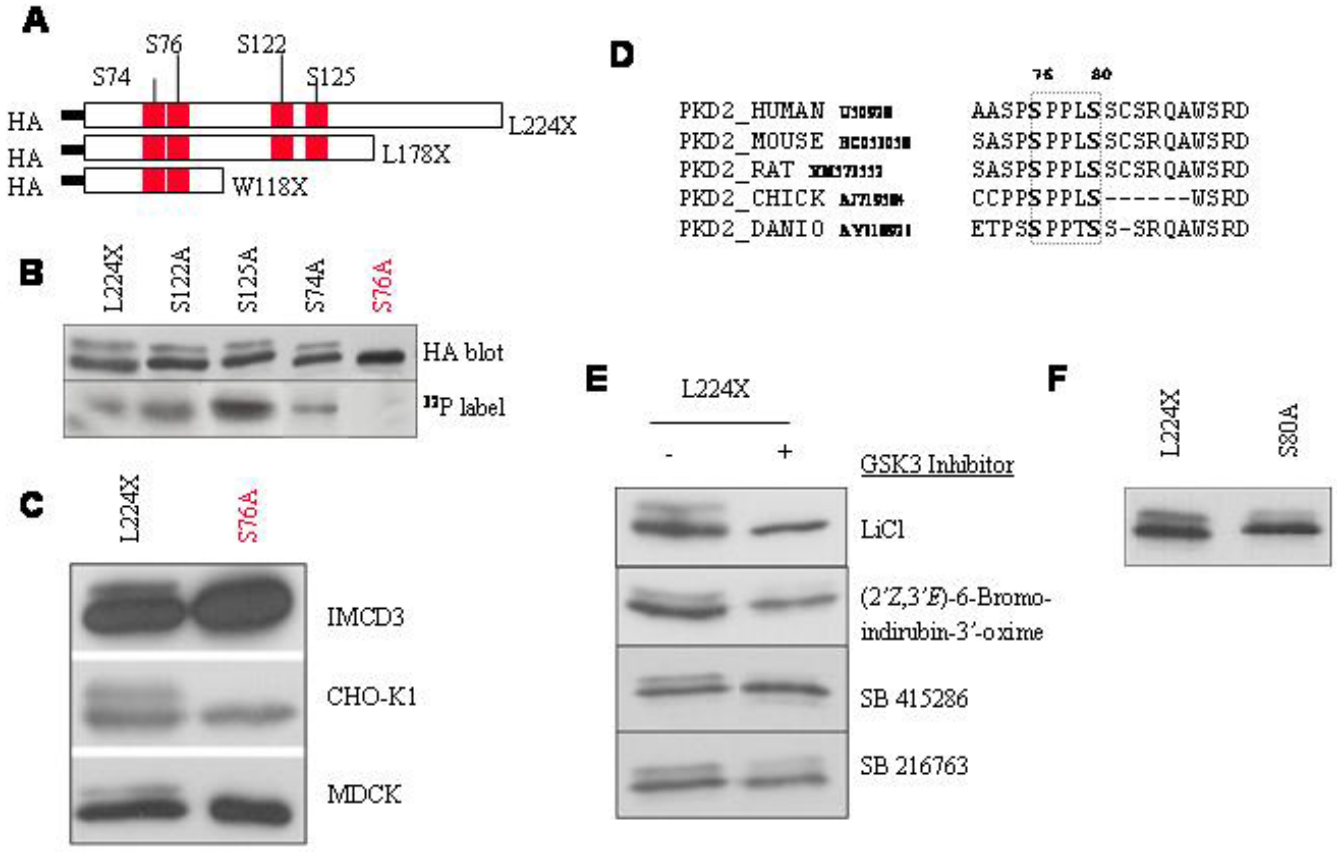

Fig 2. GSK-3 mediates phosphorylation of the N-terminus of PC2 at Ser^{76}

(A). A diagram of PC2 N-terminal constructs showing the predicted sites of phosphorylation which were mutated for phosphorylation analysis. (B). To confirm the site responsible for N-terminal phosphorylation, single site mutants of L224X were immunoblotted with an antibody directed to the HA epitope tag to detect the presence of a phosphorylated banding pattern (upper panel). Loss of the upper phosphorylated band was only seen in cells transfected with the S76A mutant construct. Immunoprecipitation of N-terminal mutants following metabolic labelling with ^{32}P in transfected HEK-293 cells (lower panel) confirmed this result demonstrating that Ser^{76} is responsible for N-terminal PC2 phosphorylation. (C). L224X and S76A mutants were transfected into IMCD3, CHO-K1 and MDCK cells. Lysates were immunoblotted with an antibody directed to the HA epitope tag to detect the presence of a phosphorylated banding pattern. Phosphorylation was absent as seen by the loss of the upper phosphorylated band in cells transfected with the S76A mutant construct but its presence in wild type controls. (D). Sequence data for various PC2 homologues aligned by Clustal W indicate that a GSK-3 recognition sequence is conserved across species down to lower vertebrates. The accession numbers for each sequence are as follows: Q13563 (Homo sapiens), O35245 (Mus musculus), XP573552 (Rattus norvegicus), Q5ZM00 (Gallus gallus), Q6IVV8 (Danio rerio). (E). To confirm that phosphorylation at Ser^{76} is mediated by GSK-3, a number of GSK-3 specific inhibitors, (i) LiCl (10mM) (ii) BIO (2′Z,3′E)-6-Bromoindirubin-3′-oxime (5 μM; Calbiochem, UK) (iii) SB 216763 (10 μM; Tocris, UK) and (iv) SB 415286 (40 μM; Tocris, UK) were added to HEK-293 cells transfected with L224X for 16 h at the concentrations indicated. Lysates were immunoblotted with an antibody directed to the HA epitope tag to detect the presence of a phosphorylated banding pattern. Phosphorylation was absent as seen by the loss of the upper phosphorylated band in cells treated with each inhibitor but present in untreated controls.

(F). HEK-293 cells were transfected with L224X or L224X containing a mutation of Ser^{80} to alanine. Lysates were immunoblotted with an antibody directed to the HA epitope tag to detect the presence of a phosphorylated banding pattern.

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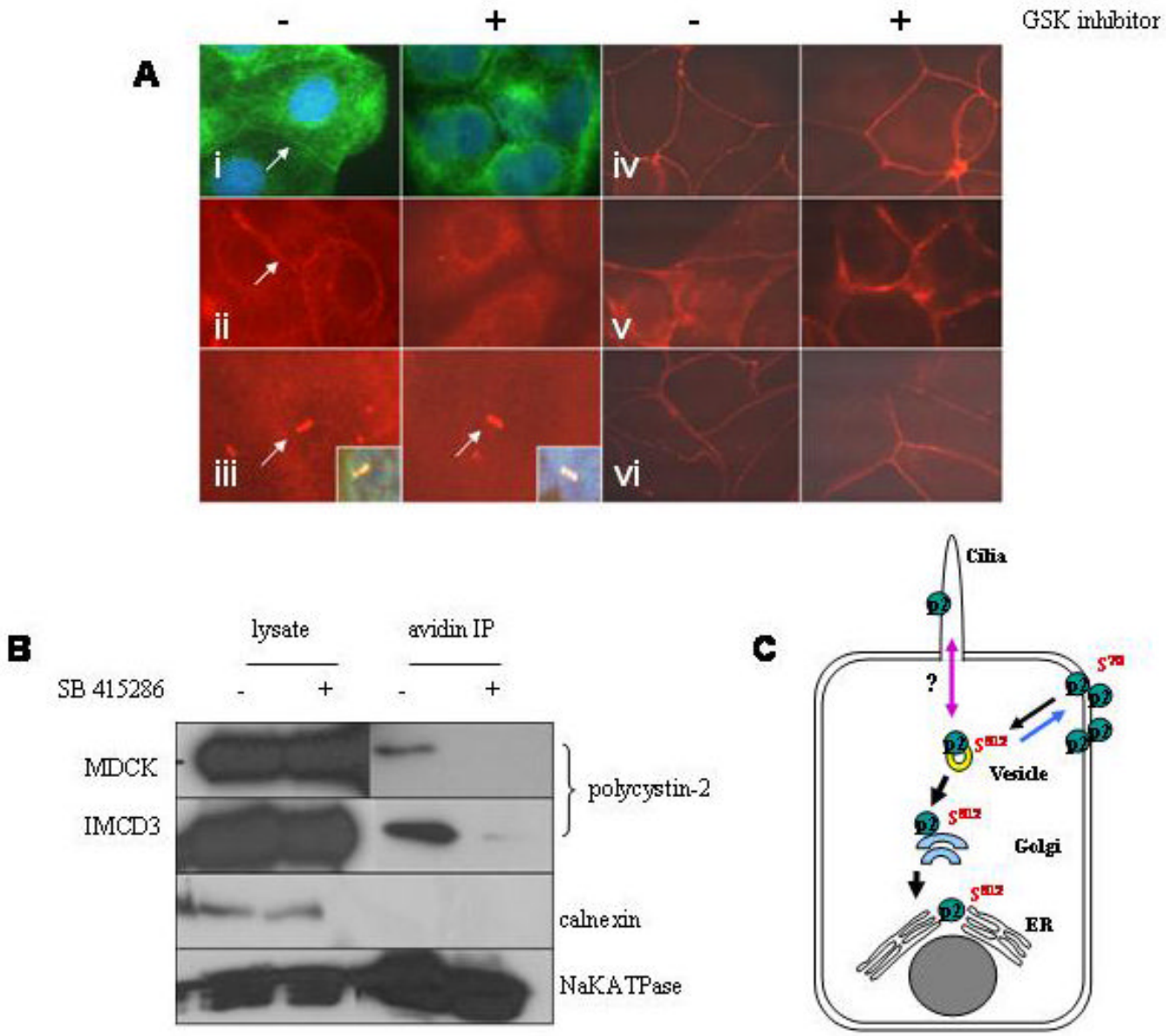

Fig 3. Inhibition of GSK-3 alters the cellular localisation of PC2

(A). Immunofluoresence staining was carried out on MDCK cells treated with 10 mM LiCl (i) or 40 μM SB 415286 (ii, iii, iv, v, vi) for 16 h compared to control untreated cells. In the presence of LiCl or SB 415286, endogenous PC2 (detected with affinity-purified p30) redistributes from a lateral plasma membrane pool (arrows) into an intracellular compartment in MDCK cells (i, ii) with no change in ciliary localisation (iii, arrows). The distribution of ZO-1 (iv), E-cadherin (v) and desmoplakin (vi) remained unchanged after GSK-3 inhibition. The insets in (iii) show colocalisation of the PC2 signal (red) with acetylated tubulin (green) confirming that cilia localisation of PC2 did not change after inhibition of GSK-3.

(B). Cell surface biotinylation was carried out on mIMCD3 and MDCK cells treated with 40 μM SB 415286 for 16 h compared to control untreated cells. Western blotting with a specific anti-PC2 antibody (1A11) was carried out on biotinylated protein bound to and eluted from streptavidin beads. The blots show that a fraction (~10%) of PC2 is present at the cell surface in control cells but is greatly reduced (~1.5%) in cells treated with the inhibitor.

Immunoblotting with antibodies directed to an ER resident protein, calnexin and a cell surface marker, Na^+K^+ATPase show that only cell surface proteins have been biotinylated and these are unaffected by treatment with the inhibitor.

(C). Diagram to illustrate the anteograde (mediated by Ser^{76}) and retrograde (mediated by Ser^{812}) trafficking of PC2 between different subcellular compartments and the lateral plasma membrane. The signals that regulate PC2 trafficking to the primary cilium are not known but appear to be independent of GSK-3 phosphorylation at Ser^{76}. PC2 is displayed as a homotetramer on the basis of biochemical characterisation and by analogy to other TRP channels (8). However, PC2 can also form heterodimers with PC1 and TRPC1 (see text).

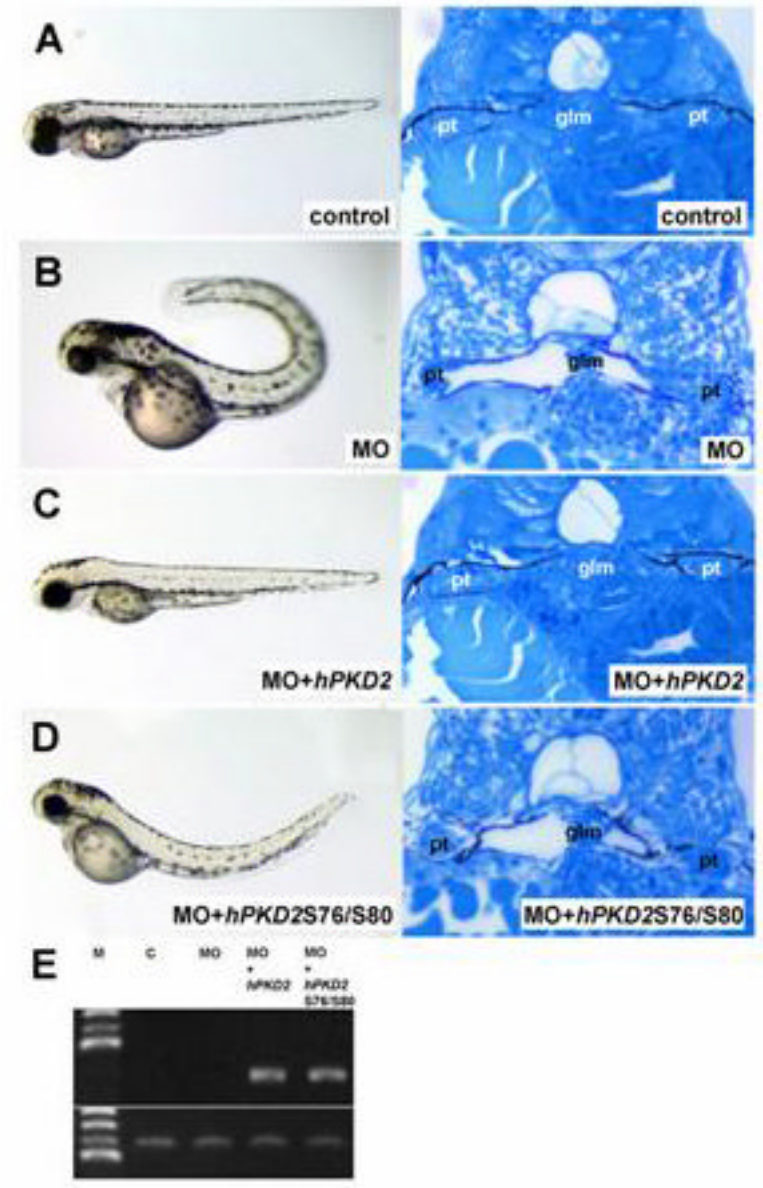

Fig 4. Human wild type but not a human *PKD2*-S76A/S80A mRNA can rescue the *pkd2* ATGMO phenotype in zebrafish

(A) 72 hpf zebrafish embryos injected with a control antisense MO have normal body morphology as described. (30)Histology section of a 72 hpf embryo pronephric kidney showing glomerulus (glm) and pronephric tubule (pt). (B) *pkd2*ATGMO injected embryos showing pronephric kidney cysts, body axis curvature and hydrocephalus. Cystic dilatation of pronephric tubules and glomerulus lined with stretched epithelium are clearly visible. (C) Co-injection of 50pg human *PKD2* mRNA suppresses formation of pronephric kidney cysts, body axis curvature and hydrocephalus. (D) Co-injection of 50pg human *PKD2*-S76A/S80A mRNA cannot rescue pronephric cysts and hydrocephalus caused by *pkd2*ATGMO. Body axis curvature is slightly rescued by human *PKD2*-S76A/S80A mRNA. (E) RT-PCR analysis for human *PKD2* (upper panel) and *β-actin* (lower panel) mRNA expression in 72 hpf embryos. DNA marker (lane M), control embryos (lane C), *pkd2*ATGMO injected embryos (lane MO), *pkd2*ATGMO and human *PKD2* mRNA injected embryos (lane MO+*hPKD2*), and

*pkd2*ATGMO and human *PKD2*-S76A/S80A mRNA injected embryos (lane MO+*hPKD2*-S76A/S80A) were analyzed with RT-PCR primers that generate a 172-bp product representing the N-terminus of human *PKD2*. Amplification of *β-actin* mRNA (lower panel) was used as a positive control.

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