Modulation of the Secretory Pathway Rescues Zebrafish Polycystic Kidney Disease Pathology

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ABSTRACT
Mutations in polycystin 1 and polycystin 2 are responsible for autosomal dominant polycystic kidney disease, the most common heritable human disease. Polycystins function as calcium ion channels, but their impact on cell physiology is not fully known. Recent findings suggest that polycystins could function in the maintenance of extracellular matrix integrity. In zebrafish, polycystin 2 knockdown induces kidney cysts, hydrocephalus, left/right asymmetry defects, and strong dorsal axis curvature. Here, we show that increased notochord sheath collagen deposition in polycystin 2–deficient embryos is directly linked to axis defects. Increased collagen II protein accumulation did not associate with increased col2a1 mRNA or a decrease in matrix metalloproteinase activity but, instead, it associated with increased expression of the endoplasmic reticulum/Golgi transport coat protein complex II Sec proteins. sec24D knockdown prevented dorsal axis curvature and kidney cystogenesis in polycystin 2 morphants. Nontoxic doses of brefeldin A also prevented the dorsal axis curvature formation in polycystin 2 morphants and curly up polycystin 2 mutants. Brefeldin A treatment after the onset of polycystin deficiency phenotypes reversed the curved axis phenotype but not kidney cyst progression. Our results suggest that polycystin 2 deficiency causes increased collagen II synthesis with upregulation of secretory pathway coat protein complex II components. Restoration of normal rates of secretory protein synthesis and secretion may be a new target in the treatment of autosomal dominant polycystic kidney disease.

Autosomal dominant polycystic kidney disease (ADPKD) is the most common heritable human disease, with an incidence of 1 in 800 births, and it is caused by mutations in polycystic kidney disease 1 (PKD1) and PKD2 encoding polycystin 1 and polycystin 2.1 ADPKD is characterized by the presence of epithelial cysts in both kidneys as well as cysts in the liver and pancreas. In addition to cystic pathology, ADPKD is also linked to abdominal hernia, and it is one of the best defined heritable disorders associated with intracranial aneurysm2 (conditions associated with both altered extracellular matrix (ECM) composition and integrity). Other manifestations of ADPKD include gastrointestinal cysts and cardiac valve defects.3 Aortic aneurysm and vascular fragility are also observed in mouse models of ADPKD.4,5 These features of ADPKD suggest that altered tissue structure or ECM integrity could play an important role in this disease; however, a mechanism linking ECM abnormalities and the cellular functions of polycystins has been elusive.

Polycystin 1 and polycystin 2 are multipass transmembrane proteins that function together as a receptor–ion channel complex,1 leading to the widely held view that primary cellular defects in polycystin–deficient cells are caused by a disruption of normal Ca2+ signaling. The polycystin 1/2 complex in apical cilia participates in mechanosensory Ca2+ signaling in response to fluid flow,6 suggesting that loss of either polycystins or cilia
structure would lead to acute loss of flow sensation and cyst formation. However, the relatively slow and sporadic development of kidney cysts in polycystin 1 and cilia protein conditional knockout models has suggested that other factors may contribute to cyst formation. Polycystin 1 and polycystin 2 are also localized to the endoplasmic reticulum (ER) membrane, where they function to regulate ER calcium stores, calcium release, and intracellular calcium homeostasis.7–9 The ER and Golgi apparatus are also the main vesicular organelles associated with secretory protein synthesis and vesicle trafficking; altered ER calcium handling in ADPKD and the involvement of multiple calcium binding proteins in vesicular trafficking suggest that ADPKD could affect secretion. Indeed, early studies of ADPKD patient cells revealed defects in ECM proteoglycan synthesis in ADPKD cells,10–13 whereas more recently, Golgi function and basolateral exocytosis have been shown to be altered in PKD1 mutant epithelial cells,14 with altered intracellular vesicle trafficking linked to mislocalization of sec6, sec8, and E–cadherin proteins. Although the data are suggestive of a link between altered secretory protein production and disease pathology, it remains to be shown that modulating the secretory pathway can impact tissue morphogenesis defects associated with ADPKD.

We and others have modeled ADPKD in the zebrafish by knockdown or mutation of orthologous pkd genes.15–17 Polycystin loss of function results in left–right asymmetry, kidney cysts, hydrocephalus, and a strong dorsal axis curvature. Dorsal axis curvature is a fully penetrant phenotype associated with overaccumulation of collagen type II protein in the notochord sheath.18 Here, we show that accumulation of collagen type II in the notochord sheath is a consequence of an increased protein production and secretion linked to upregulation of the cytoplasmic coat protein II (COPII) components sec23B, sec24C, and sec24D. Inhibition of the secretory pathway by knockdown of sec24D or chemical inhibition with brefeldin A prevents the dorsal axis curvature and cyst formation observed in polycystin 2–deficient embryos. Our data suggest a causative role for increased ECM synthesis and secretion in the initiation of pkd2 deficiency phenotypes and raise the possibility that vesicle trafficking may be a target to ameliorate disease pathogenesis in human ADPKD patients.

RESULTS
Altered Collagen Type II Accumulation Mediates Axis Defects in pkd2-Deficient Embryos
Polycystin 1a/b and polycystin 2 deficiency in zebrafish results in kidney cysts, hydrocephalus, and strong dorsal axis curvature (Figure 1, A and B, Supplemental Figure 1).15,16,18 Dorsal axis curvature is the most penetrant and consistent phenotype, affecting almost 100% of zebrafish pkd2 morphants/mutants, and it is associated with overexpression of notochord sheath collagen type II.18 We have previously shown that complete collagen type II protein knockdown rescued the pkd2 axis curvature phenotype, suggesting that collagen type II overproduction may be causally linked to axis curvature or alternatively, that collagen type II may simply be required to stabilize an altered axial structure induced by other factors in pkd2-deficient embryos. To distinguish between these possibilities, we used lower doses of col2a1a morpholino and attempted to restore collagen type II protein to near wild-type levels in pkd2 morphants. Partial col2a1a knockdown almost completely rescued dorsal axis curvature (Figure 1, A–C). Collagen type II immunostaining revealed that partial col2a1a knockdown successfully restored the amount of collagen type II to wild-type levels in the notochord sheath of pkd2 morphants as shown in longitudinal views of the notochord (Figure 1, D–L). Phenotype quantification showed that nearly all (96.5%) of the pkd2 morphants exhibited moderate or severe dorsal axis curvature at 48 hours postfertilization (hpf), whereas embryos coinjected with col2a1a and pkd2 morpholinos showed only mild or no curvature (Figure 1M). Knockdown of col2a1a in the ventrally curved kidney cyst mutant double bubble19 had no effect on mutant curvature (Supplemental Figure 2). Taken together, these results suggest that matrix overproduction is directly linked to the axis curvature phenotype and that this mechanism is specific to pkd2 deficiency.

Altered Collagen Type II Accumulation Occurs Post-Transcriptionally
To test whether collagen overaccumulation in the notochord sheath of pkd2 morphants was caused by increased collagen mRNA expression or other mechanisms, we assessed col2a1a and col9a2 expression by quantitative RT-PCR. Surprisingly, expression of col2a1a (Figure 1N) and col9a2 mRNA (Figure 1O) was similar in pkd2 morphants compared with wild-type embryos at 24 and 48 hpf, suggesting a post-transcriptional mechanism for overaccumulation of collagen type II protein. Consistent with this idea, low, relatively nontoxic doses of the protein synthesis inhibitor cycloheximide (20 μg/ml) were sufficient to prevent the pkd2 axis curvature phenotype (Supplemental Figure 3). These results suggested that overaccumulation of notochord collagen II protein was most likely linked to enhanced collagen translation, secretion, or reduced degradation.

Collagen Type II Accumulation Is Linked to Increased Expression of COPII Components
Collagen mRNA translation, fibril assembly, and secretion are subject to multiple processing steps that could affect overall collagen abundance or integrity of the ECM. A critical step of collagen processing is its vesicular trafficking from the ER, where it is synthesized, to the Golgi apparatus for secretion. The COPII complex that consists of multiple Sec proteins mediates ER to Golgi vesicle traffic. Interestingly, quantitative RT-PCR expression analysis of mRNA encoding the COPII components sec23A, sec23B, sec24C, and sec24D indicated that the level of sec23B, sec24C, and sec24D mRNAs (Figure 2, B–D) was...
significantly increased in *pkd2*-deficient embryos compared with wild type at 48 hpf, whereas *sec23A* mRNA expression was not affected (Figure 2A). These data suggest that accumulation of collagen type II could be linked to an increased rate of secretion. Other factors, such as expression of the collagen crosslinking lysyl oxidase genes *lox*, *lox1*, and *lox1b* (Supplemental Figure 4, A–C) or the crosslinked status of collagen type II protein itself (Supplemental Figure 4, D–F), were not affected. Also, it is unlikely that decreased collagen degradation could account for increased collagen type II accumulation; matrix metalloproteinase (MMP) mRNA expression did not decrease in *pkd2* morphants (Supplemental Figure 5), and multiple different MMP (GM6001, doxycycline, and ascorbic acid) and proteasome (MG132 and MLN4924) inhibitors did not induce axis curvature in wild-type embryos (data not shown). Increased accumulation of collagen type II protein could, however, be detected in Western blots of *pkd2* morphants, confirming our immunostaining results (Supplemental Figure 4, D–F).

Figure 1. Collagen type II overexpression plays a direct role in dorsal axis curvature in *pkd2*-deficient embryos. (A) Wild-type embryo and (B) *pkd2* morphant (*pkd2* MO) at 48 hpf; (C) *col2a1a* knockdown in *pkd2* morphants prevents the dorsal axis curvature. (D–L) Confocal images showing collagen type II immunostaining (red) and wga labeling (green) in the notochord of a (D and G) wild-type embryo, (E and H) *pkd2* morphant, and (F and I) *col2a1a/pkd2* double morphants (*pkd2*+*col2a1a* MO) at 30 hpf. (J–L) Merged images of collagen type II and wga; (D–F) *col2a1a* partial knockdown in *pkd2* morphants reduces the collagen type II level close to wild-type level. Stacks were acquired through the whole notochord, and images from the middle of the notochord were selected. (M) Quantification of the percentage of embryos showing no curvature, mild curvature, moderate curvature, and severe curvature in the different experimental groups from a single representative experiment (of four replicates). Wild-type embryos (*n*=89); *pkd2* morphants (*n*=57); *pkd2*+*col2a1a* morphants (*n*=101). (N and O) Quantitative RT-PCR measurements of *col2a1a* and *col9a2* mRNA abundance showing no difference in wild-type embryos (white bars) and *pkd2* morphants (black bars) at 24 and 48 hpf. Expression is shown relative to the *EIF1a* mRNA level (*n*=4 per group). Differences in *col2a1a* mRNA levels were not statistically significant. A small increase in *col9a2* mRNA was significant at 24 hpf (**P=0.003) but not 48 hpf. WT, wild-type. Scale bars, 10 μm.
Knockdown Prevents the Dorsal Axis Curvature and Cyst Formation in \( \textit{pkd2} \)-Deficient Embryos

To determine if increased Sec gene expression might be linked to the increased collagen production observed in \( \textit{pkd2} \)-deficient embryos, we knocked down \( \textit{sec24D} \) in \( \textit{pkd2} \) morphants and monitored dorsal axis curvature as a readout of altered ECM. Significantly, the severity of curvature in the \( \textit{pkd2/sec24D} \) double morphants was reduced compared with \( \textit{pkd2} \) morphants at 48 hpf (Figure 2, E–G). Quantification revealed that 86% of \( \textit{pkd2} \) morphants had severe curvature compared with 38% of the double morphants, indicating that the secretory pathway plays a crucial role in this phenotype (Figure 2H). To test if increased sec gene expression was also linked to pronephric cyst formation, we monitored kidney cyst formation in the \( \text{Tg(wt1b:GFP)} \) line after knocking down \( \textit{sec24D} \) in \( \textit{pkd2} \) morphants. Cyst formation was decreased in double morphants compared with \( \textit{pkd2} \) knockdown alone (Figure 2, I–K).

**Figure 2.** Overexpression of sec genes contributes to dorsal axis curvature and cystogenesis in \( \textit{pkd2} \)-deficient embryos. (A–D) Quantitative RT-PCR measurements of sec genes showing an increased expression of (B) \( \textit{sec23B} \), (C) \( \textit{sec24C} \), and (D) \( \textit{sec24D} \) in \( \textit{pkd2} \) morphants (black bars) compared with wild-type embryos (white bars) at 48 hpf (n=4 per group; *P<0.05; **P<0.01; ***P<0.001). (E) Wild-type embryo, (F) \( \textit{pkd2} \) morphant, and (G) \( \textit{sec24D} \) knockdown in \( \textit{pkd2} \) morphant at 48 hpf. (H) Quantification of the number of embryos with severe dorsal axis curvature in three independent experiments revealed that \( \textit{sec24D} \) knockdown can prevent the dorsal axis curvature in \( \textit{pkd2} \) morphants. Wild-type embryos and \( \textit{sec24D} \) morphants never displayed any curvature. Wild type (n=512); \( \textit{sec24D} \) morphants (n=175); \( \textit{pkd2} \) morphants (n=179); \( \textit{pkd2+sec24D} \) morphants (n=129). *P=0.04. (I–K) Dorsal view of zebrafish pronephros in a (I) control \( \text{Tg(wt1b:GFP)} \) embryo, (J) \( \text{Tg(wt1b:GFP)} \) \( \textit{pkd2} \) morphant, and (K) \( \text{Tg(wt1b:GFP)} \) \( \textit{sec24D} \) MO in \( \textit{pkd2} \) morphants at 48 hpf. (L) The quantification of three independent experiments shows that the injection of \( \textit{sec24D} \) morpholino in \( \textit{pkd2} \) morphants decreases cyst formation compared with \( \textit{pkd2} \) morphants. Wild type (n=41); \( \textit{sec24D} \) morphants (n=31); \( \textit{pkd2} \) morphants (n=43); \( \textit{pkd2+sec24D} \) morphants (n=41). MO, morpholino oligo–injected; WT, wild-type.
I–K). Cyst quantification revealed that 49% of pkd2 morphants exhibited cysts compared with 17% of the double morphants (Figure 2L). Cyst rescue was not associated with any increase in apoptosis in double morphants (Supplemental Figure 6, A–D).

**Brefeldin A Prevents Dorsal Axis Curvature in pkd2 Mutants and Morphants**

Brefeldin A (BFA) blocks ER to Golgi vesicular traffic by stabilizing the Arf-GDP:ArfGEF intermediate and preventing Arf cycling.21,22 High doses of BFA (10 µg/ml) completely block ER/Golgi traffic in zebrafish embryos, inducing the ER stress response and apoptosis.23 We reasoned that low, nontoxic doses of BFA might reduce the rate of vesicle trafficking without inducing cell stress. We, therefore, treated wild-type zebrafish embryos and pkd2 morphants with low to intermediate doses of BFA (from 0.5 to 2 µg/ml) and monitored collagen accumulation. Collagen type II is primarily localized in the notochord sheath ECM in wild-type embryos and pkd2 morphants at 30 hpf. (Figure 3, A, B, E, F, I, J, M, and N). However, after BFA treatment, collagen accumulated in a vesicular pattern, indicating that low-dose BFA inhibits secretion (Figure 3, C, D, G, H, K, L, O, and P) and can reduce collagen type II accumulation in the ECM of pkd2 morphants to wild-type levels (Figure 3, D, H, L, and P). Notably, collagen II–containing vesicles were always much larger in pkd2 morphants, consistent with an ECM overproduction phenotype (Figure 3D). Wild-type embryos treated with BFA exhibited a shortened body axis, especially at the highest dose tested (2 µg/ml) (Supplemental Figure 7), consistent with previous studies reporting a correlation between disrupted collagen secretion and reduced body length.24,25 Strikingly, early (24 hpf) BFA treatment of pkd2 morphant embryos resulted in a dose-dependent decrease in dorsal axis curvature at 48 hpf (Figure 4, A, B, E, and F), with the highest BFA doses tested nearly completely preventing development of axis curvature (Figure 4D). Another ER/Golgi transport small molecule inhibitor, CI-976, also induced a significant dose-dependent rescue of the pkd2 axis curvature phenotype (Supplemental Figure 8).

To confirm the effects of BFA in a genetic model of pkd2 deficiency, we treated embryos from an incross of the zebrafish pkd2 mutant curly up (cup)17 with BFA. Untreated cup embryos from clutches of incrossed heterozygotes exhibited the typical Mendelian ratio (25%) of moderate to severe dorsal axis curvature (Figure 4, C and H, upper panel). BFA treatment eliminated severe axis curvature and induced a significant shift to the mild curvature class (Figure 4, G and H, lower panel). These results confirm that modulation of the secretory pathway can rescue pkd2 mutant phenotypes and further suggest causal links between increased collagen synthesis, increased secretion, and pkd pathology.

To test whether BFA treatment could restore normal morphology after axis curvature had started to form, we initiated BFA treatment at 30 hpf, when more than 60% of pkd2 morphants already exhibit dorsal axis curvature. Strikingly, addition of BFA at 30 hpf reversed axis curvature pathology (Figure 4, I–K), such that after 3 hours of BFA treatment, only 17% of pkd2 morphants exhibited curvature compared with 61% before treatment (Figure 4L). The effect persisted at 48 hpf, when only 27% of pkd2 morphants treated with BFA displayed curvature (Figure 4L). The results show that BFA treatment cannot only prevent development of axis curvature but also, restore a straight axis in previously curved embryos.

**BFA Reduces Kidney Cyst Formation in pkd2 Morphants**

To test whether BFA treatment could also prevent pkd2-associated cystogenesis, we quantified cysts in Tg(wt1b:GFP); pkd2 morphants treated with BFA (2 µg/ml). Similar to the effect of sec24D knockdown, kidney cyst formation was reduced in BFA-treated pkd2 morphants compared with nontreated

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**Figure 3.** BFA blocks collagen type II secretion in pkd2 morphants. Confocal images showing (A–D) collagen type II immunostaining (red) and (E–H) wga labeling (green) in the notochord of a (A and E) wild-type embryo, (B and F) pkd2 morphant, (C and G) BFA-treated wild-type embryo, and (D and H) BFA-treated pkd2 morphant at 30 hpf. (I–L) Merged images of collagen type II and wga. (A and B) Collagen type II staining was localized in the notochord sheath in wild-type and pkd2 morphants, with a higher expression in pkd2 morphants, whereas (C and D) it accumulates in a vesicular pattern in BFA-treated embryos, showing that BFA inhibits collagen type II sheath accumulation close to wild-type levels in pkd2 morphants. Arrowheads in A–D designate the notochord sheath ECM. (M–P) Line scan analysis of A–D (at the yellow lines in A–D) shows (N) increased collagen II immunoreactivity in pkd2 morphants and (O and P) reduction in notochord sheath collagen II (arrowheads) in BFA-treated embryos. Confocal stacks were acquired of the whole notochord, and images from the middle of the notochord were selected. Scale bars, 10 µm. MO, morpholino oligo–injected; WT, wild-type.
pkd2 morphants (15.7% versus 43%) (Figure 5, A–D). The dose of BFA used in these experiments was not sufficient to induce general apoptosis26,27 (Supplemental Figure 5), indicating that the reduction in cyst formation was not caused by generally toxicity.

The reduction in cyst frequency by early treatment with BFA suggested that modulating the secretory pathway may block critical cyst initiating events or alternatively, that BFA may alter the progression of cyst development. We, therefore, tested the efficacy of BFA in cyst inhibition when it was added at a later stage in development. BFA treatment at 30 hpf did not significantly prevent cystogenesis compared with non-treated pkd2 morphants (Figure 5, E–G). In this experiment, we found that 37% of BFA-treated pkd2 morphants

Figure 4. BFA treatment prevents dorsal axis curvature in pkd2-deficient embryos. (A) Wild-type embryo, (B) pkd2 morphant, and (C) cup embryo at 48 hpf. (E–G) Treatment with BFA (E) does not induce curvature in wild-type embryos but reduces dorsal axis curvature in (F) pkd2 morphants and (G) cup embryos when added at 2 μg/ml at 24 hpf. (D) Percentage of embryos with severe curvature in pkd2 morphants at 48 hpf (n=3 independent experiments). (H) Percentage of embryos with mild curvature (light gray bars), moderate curvature (dark gray bars), and severe curvature (black bars) in cup embryos (upper panel) and BFA-treated cup embryos (lower panel) at 48 hpf (n=3 independent experiments). pkd2 morphants (n=71); pkd2 morphants+BFA (n=57 at 0.5 μg/ml, n=75 at 1 μg/ml, n=73 at 1.5 μg/ml, n=60 at 2 μg/ml); cup mutants (n=298); cup mutants+BFA (n=222). The increase in pkd2 morphant embryos with mild curvature in the BFA sample was statistically significant (*P=0.05). (I) Wild-type embryo, (J) pkd2 morphant, and (K) BFA-treated pkd2 morphant at 48 hpf. (L) Percentage of embryos with moderate and severe curvature at 48 hpf in pkd2 morphants (black line) and pkd2 morphants treated with BFA at 30 hpf (red line). The quantification of two independent experiments at 33 hpf and three independent experiments at 48 hpf shows that BFA treatment can rescue preexisting curvature in pkd2 morphants. Wild type (n=270 at 30 hpf, n=73 at 33 hpf, n=71 at 48 hpf); pkd2 morphants (n=210 at 30 hpf, n=65 at 33 hpf, n=57 at 48 hpf); wild type+BFA (n=63 at 30 hpf, n=57 at 48 hpf); pkd2 morphants+BFA (n=68 at 33 hpf, n=53 at 48 hpf). *Percentage with severe curvature in BFA treated at 33 and 48 hpf was significantly different from untreated pkd2 morphants at 33 hpf; **percentage with severe curvature in BFA treated at 33 and 48 hpf was significantly different from untreated pkd2 morphants at 48 hpf; ***percentage with severe curvature in BFA treated at 33 and 48 hpf was significantly different from the starting time point in both cases. MO, morpholino oligo–injected; WT, wild-type.
were cystic compared with 43% of nontreated pkd2 morphants (Figure 5H). This result suggests that BFA is most likely acting at the time of cyst initiation and not, for instance, blocking transport of proteins that are required to maintain cystic distension.

DISCUSSION

Despite advances in our understanding of the functions of polycystins, mechanisms underlying ADPKD pathogenesis remain unclear. The polycystin complex is proposed to function in multiple cellular contexts, including the primary cilium as a mechanosensor,\(^6\) the ER as a calcium release channel,\(^7,8,28\) and the cell membrane as an adhesion complex.\(^29–32\) Different features of ADPKD pathology, including failed flow sensing, increased fluid secretion, and cell proliferation, may well involve polycystin function in different subcellular locations. In addition to cystic epithelial pathology in the kidney, pancreas, and liver, ADPKD is linked to changes in ECM composition in multiple organs.\(^4,33–35\) Increased cell adhesiveness, decreased cell migration, and abnormal expression of integrins have also been observed in ADPKD cells.\(^29,36\) However, it remains unclear whether these pathologies are primary in disease initiation or a secondary consequence of tissue pathology. Our previous work suggested that the most penetrant zebrafish PKD phenotype, dorsal axis curvature, was caused by notochord deformity induced by abnormal expression of ECM collagen type II.\(^18\) Using axis curvature as a readout of pkd2 deficiency, we show here that polycystin2 knockdown in zebrafish causes overproduction of collagen type II protein in the notochord sheath. Moreover, reestablishing wild-type levels of collagen type II protein in the notochord ECM restores a normal axis morphology, supporting the idea that collagen overproduction plays a causal role in zebrafish PKD axis curvature. Collagen overproduction was also associated with increased expression of secretory pathway COPII components. Sec protein knockdown or chemical inhibition of ER to Golgi transport was also sufficient to reverse PKD axis curvature and kidney cyst formation, highlighting the secretory pathway as a potentially novel target for treating PKD.

Increased collagen type II protein production in pkd2-deficient embryos could be caused by several mechanisms, including transcriptional or translational regulation of collagen expression. Whole-embryo quantitative RT-PCR mRNA quantification showed no difference in overall col2a1a or col9a2 mRNA expression between pkd2-deficient and wild-type...
embryos, suggesting a post-transcriptional mechanism. Recently, we determined that our prior findings of increased *col2a1a* or *col9a2* mRNA expression (by *in situ* hybridization) in *pkd2*-deficient embryos could be accounted for by increased embryo sensitivity to proteinase treatment used in the *in situ* hybridization protocol and enhanced *in situ* probe penetration (data not shown). These results are consistent with a general ECM defect in *pkd2*-deficient embryos that renders them more fragile and permeant to probes. Equivalent expression of collagen type II mRNA coupled with increased protein accumulation in *pkd*-deficient embryos points strongly to differences in translation or post-translational collagen type II processing as the cause of axis curvature in *pkd* morphant zebrafish. General inhibition of MMPs or the proteasome was not sufficient to induce axis defects, suggesting a principle role for increased collagen production in driving the zebrafish *pkd* phenotype.

The simplest interpretation of our data may be that polycystin 2 activity in the ER either directly or indirectly regulates secreted protein translation or vesicle trafficking. The altered expression of *sec23B*, *sec24C*, and *sec24D* that we observe supports the idea that ER to Golgi secretory traffic may be generally increased in *pkd* morphants. The role of *Sec24D* in the secretion of collagen type II has already been shown in the zebrafish. Bulldog and feelgood mutants, in which *sec24D* is disrupted, fail to secrete collagen type II, leading to abnormal cranial morphogenesis and shorter body length, likely because of notochord defects. Previous studies have reported altered secretion of heparin sulfate proteoglycans and defective basolateral vesicle trafficking in ADPKD cells. Polycystin 2 itself interacts with and is regulated by Syntaxin5, an essential regulator of vesicle trafficking. Consistent with a role for polycystin 2 specifically in the ER, expression an ER-retained mutant polycystin 2 protein in zebrafish *pkd* morphants has been shown to reverse the dorsal axis curvature phenotype that we have linked to excess collagen production. Could polycystin 2 deficiency directly modify the secretory pathway? PKD mutant cells are known to exhibit reduced agonist-stimulated calcium release from the ER and in some cases, show enhanced amounts of ER calcium. In localized transport between the ER and Golgi, calcium binding proteins regulate vesicle trafficking. For instance, calcium regulates the binding between Sec31A and Apoptosis-linked gene 2, a penta-EF-hand protein that regulates secretion by stabilizing the COPII complex at the ER. Additional studies will be required to determine whether altered calcium release from the ER in *pkd*-deficient embryos could influence vesicle transport rates or the specific vesicle cargo sorting processes required for collagen secretion.

Regardless of whether *pkd* knockdown directly or indirectly affects secretion, our finding that *sec24D* knockdown or BFA treatment was sufficient to prevent *pkd* phenotypes in zebrafish argues strongly that altered protein secretion is one critical component of *pkd* pathology. BFA treatment resulted in a dose-dependent rescue of dorsal axis curvature in both *pkd2* morphants and *cup pkd2* mutants and prevented kidney cyst formation in *pkd2* morphants. At the concentrations used, BFA did not induce apoptosis or major defects in zebrafish embryos, except a shortened body length. Most importantly, we showed that BFA treatment could straighten embryos that already had a mild dorsal axis curvature before treatment, suggesting that reversal of collagen overproduction could allow realignment of ECM to a normal axial morphotype. However, BFA treatment was unable to rescue cyst formation in *pkd2* morphants after cysts were formed. The results suggest that ECM defects could play a role in the initiation of cystogenesis and dorsal axis curvature but perhaps, could not play a role in progression of the cystic phenotype.

We have focused here on the secretory pathway as a potential target to reverse collagen overaccumulation in the ECM of *Pkd2*-deficient embryos. However, it is also clear from our work that collagen overaccumulation must also involve an increased rate of *col2a1a* mRNA translation. The observed changes in COPII component gene expression could be a compensatory event to support increased secretion. Although the Tor pathway is known to be activated in ADPKD cells and could, in principle, signal increased protein synthesis, we did not observe an increase in ribosomal phospho-S6 protein levels (Supplemental Figure 9), a readout of Tor signaling. Although additional studies will be required to determine the mechanism of increased collagen translation, our finding that modulation of the secretory pathway prevented even reversed some ADPKD phenotypes in zebrafish suggests that the COPII complex may be a new target in the treatment of ADPKD.

**CONCISE METHODS**

**Zebrafish Lines**

Wild-type TüAB and Tg(*wt11b:GFP*) transgenic zebrafish were maintained and raised as described previously. Dechorionated embryos were kept at 28.5°C in E3 solution with or without 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to suppress pigmentation and staged according to hpf.

**Morpholino Antisense Oligonucleotide Injections**

Wild-type (TüAB) or WT1-GFP embryos at the one- to two-cell stages were microinjected with 4.6 nl 0.05 mM *col2a1* or 0.25 mM *pkd2* or *sec24D* antisense morpholino oligonucleotide solution (Gene Tools LLC) in 200 mM KCl with 0.1% Phenol Red using a nanoject2000 microinjector (World Precision Instruments). Morpholino sequences were

- *pkd2* exon 12: 5′-caggtgtgatgttacctggaactc-3′;
- *col2a1a* exon 1: 5′-tgaaaaactccaacttacggtcatc-3′; and
- *sec24D* 5′ untranslated region: 5′-cttacctgtatactcttcttcatcc-3′.
The control morpholino sequence was 5′-cgtccattgtgtaaagtgttaacca-3′ (irrelevant sequence).

**Quantitative RT-PCR**

RNAs were obtained from 24-, 48-, or 72-hpf embryos using the Qiagen RNeasy mini kit. RNAs were treated with DNase (DNase I, RNase-free; Roche Diagnostics) and reverse-transcribed using oligo dT according to the manufacturer’s protocols (Superscript III; Invitrogen). Quantitative RT-PCR was performed using SYBR Green. Primer sequences are listed in Table 1. Each RNA sample is obtained from 10 embryos by quantitative RT-PCR. Gene expression was normalized with the housekeeping gene EIF1α.

**Collagen Crosslinking**

Collagen type II was extracted from 500 desiccated wild-type embryos or pdk2 morphants with 4 M guanidine HCl and 0.05 M Tris·HCl (pH 7.4) with 2 mM PMSF added as a proteases inhibitor for 48 hours at 4°C. The washed residue was digested with pepsin at 4°C and rocked for 24 hours, and the reaction was then stopped by the addition of 0.7 M NaCl. The samples were run on a 6% SDS-PAGE gel and trans-blotted to a polyvinylidene difluoride membrane for Western blot detection of collagen type II chains with a monoclonal antibody 1C10 that recognizes a sequence-specific epitope in the α1(II) triple helical domain (residues 934–945).[^50]

**Drug and Small Molecule Treatments**

Collagen crosslinking was inhibited by treating embryos in E3 water with the lysyl oxidase inhibitor β-aminopropionitrile (30 mM) from postgastrulation (6–8 hpf) to 48 hpf. The secretary pathway from the ER to the Golgi apparatus was inhibited using BFA or CI-976. BFA was dissolved in ethanol and used at 0.5, 1, 1.5, and 2 μg/ml in E3 egg water containing 0.25% ethanol. CI-976 was dissolved in DMSO and used at 1–10 μM in E3 egg water containing 0.5% DMSO. Embryos were treated with BFA from 24 or 30 to 48 hpf as indicated or CI-976 from 24 to 30 hpf. Concentrations of MMP inhibitors used (treatment starting at gastrulation) were 10 μM GM6001, 10 μM, 100 μM, and 1 mM doxycycline, and 50, 100, 200, and 500 μM ascorbic acid in E3 water. Proteasome inhibitors MG132 and MLN-4924 were used at 10, 20, and 50 μM in E3 water.

**Quantification of Dorsal Axis Curvature**

Embryos were sorted as having a curvature of less than 90° (mild), a curvature of more than 90° (moderate), or a curvature with a tail crossing the body axis (severe) at 48 hpf (Supplemental Figure 1).

**Statistical Analysis**

A t test was performed to compare the different experimental groups (*P<0.05; **P<0.01; ***P<0.001).

**Western Blotting**

Anti–phospho-S6 rabbit monoclonal (Cell Signaling Technology) was used at 1:2000 dilution and standardized using anti-S6 mouse monoclonal (Cell Signaling Technology) used at 1:500 dilution.

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**DISCLOSURES**

None.

**REFERENCES**


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