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REVIEW

The molecular structure and function of fibrocystin, the key gene product implicated in autosomal recessive polycystic kidney disease (ARPKD)

Travis A K Bannell[1,2] | **Joseph J B Cockburn**[1,2]

[1]Astbury Centre for Structural and Molecular Biology, University of Leeds, Leeds, UK

[2]School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK

Correspondence
Joseph J B Cockburn, Astbury Centre for Structural and Molecular Biology, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK. Email: j.j.b.cockburn@leeds.ac.uk

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Abstract

Autosomal recessive polycystic kidney disease is an early onset inherited hepatorenal disorder affecting around 1 in 20,000 births with no approved specific therapies. The disease is almost always caused by variations in the polycystic kidney and hepatic disease 1 gene, which encodes fibrocystin (FC), a very large, single-pass transmembrane glycoprotein found in primary cilia, urine and urinary exosomes. By comparison to proteins involved in autosomal dominant PKD, our structural and molecular understanding of FC has lagged far behind such that there are no published experimentally determined structures of any part of the protein. Bioinformatics analyses predict that the ectodomain contains a long chain of immunoglobulin-like plexin-transcription factor domains, a protective antigen 14 domain, a tandem G8-TMEM2 homology region and a sperm protein, enterokinase and agrin domain. Here we review current knowledge on the molecular function of the protein from a structural perspective.

KEYWORDS
ARPKD, ciliopathy, extracellular-like vesicle, fibrocystin, kidney, polycystic

1 | INTRODUCTION

Autosomal recessive polycystic kidney disease (ARPKD; OMIM 263200) is a severe, early onset inherited disorder affecting around 1 in 20,000 live births (Gunay-Aygun et al., 2006; Zerres et al., 1998, 2003). The principal manifestation of the disease is congenital renal insufficiency and greatly enlarged, echogenic kidneys, resulting from fusiform dilation of the collecting ducts. Around 30% of ARPKD births die in the first four weeks of life due to respiratory insufficiency or infection (Capisonda et al., 2003; Guay-Woodford & Desmond, 2003; Luthy & Hirsch, 1985). Affected infants who survive the neonatal period face continued health issues; more than 50% of patients progress to end-stage renal disease in their first 10-years, and ~30% of all patients require a kidney transplant by the age of 20 (Burgmaier et al., 2019; Gunay-Aygun et al., 2010; Telega et al., 2013). In addition, many ARPKD patients suffer from progressive congenital hepatic fibrosis (CHF), causing portal hypertension. Extrarenal manifestations affecting the spleen, brain and heart may also occur (Chinali et al., 2019; Gunay-Aygun et al., 2013; Hartung et al., 2014; Jahnukainen et al., 2015). APRKD bears resemblance to the more common autosomal dominant polycystic kidney disease (ADPKD), with overlapping manifestations (Table 1). Prenatal presentation of enlarged kidneys is

TABLE 1 Comparison of autosomal recessive polycystic kidney disease (ARPKD) and autosomal dominant polycystic kidney disease (ADPKD).

Characteristic	ARPKD	ADPKD	Citation
OMIM accession	263200	173900	N/A
Disease loci	*PKHD1*, possibly *DZIP1L*	*PKD1*, *PKD2*	Bergmann (2015), Lu et al. (2017), Onuchic et al. (2002)
Prevalence	~1:20,000 live births	~1:2000 live births	Zerres et al. (1998)
Onset	From birth, early infancy, rarely in adulthood	20–40 years of age, ~2% infancy	Bergmann (2015), Roy et al. (1997)
Inheritance (Risk for Siblings); Males:Females	Autosomal recessive (25%), 1:1	Autosomal dominant (50%), 1:1	Bergmann (2015), Guay-Woodford and Desmond (2003), Zerres et al. (1998)
Renal presentation	Enlarged, echogenic kidneys, poor corticomedullary differentiation, multiple small cysts, fusiform dilation of collecting ducts	Normal sized kidneys, distinguishable corticomedullary differentiation, variable larger cysts which increase kidney volume, rapid decline in glomerular filtration rate in adulthood	Hartung and Guay-Woodford (2014), Lanktree and Chapman (2017)
Renal cystogenesis	Dilated collecting ducts and distal tubules, tiny (<2 mm), 'salt and pepper' pattern in ultrasound	Across the nephron, cysts gradually increase in size and can reach several cm in diameter	Bergmann (2015), Grantham (1983), Iorio et al. (2020)
Hepatic presentation	Congenital hepatic fibrosis, Caroli syndrome	Biliary cysts	Bergmann (2015), Guay-Woodford and Desmond (2003)
Primary clinical manifestations	Systemic and portal hypertension, end-stage renal disease, pain, hyponatremia, hypocitraturia, cholangitis and varices	Systemic hypertension, end-stage renal disease, acute abdominal pain, haematuria and nephrolithiasis	Hartung and Guay-Woodford (2014), Lanktree and Chapman (2017)
Affected organ systems	Kidney, liver, lungs, spleen, brain and heart	Kidney, liver, pancreas, thyroid, brain and heart	Chinali et al. (2019), Gunay-Aygun et al. (2013), Halvorson et al. (2010), Hartung et al. (2014), Jahnukainen et al. (2015)
Treatment	Incurable, symptomatic relief, eventual renal and/or hepatic transplant; no licenced therapeutic	Incurable, symptomatic relief, eventual renal and/or hepatic transplant; tolvaptan is FDA approved to reduce cyst growth and renal decline	Burgmaier et al. (2019), Lanktree and Chapman (2017), Müller et al. (2022), Torres et al. (2012)
Prognosis	The most severely affected patients die prenatally; ~30% patients die perinatally; patients who survive the perinatal period variably live into early adulthood	Rare early-presenting cases have very poor prognosis, ~70% patients enter end-stage renal disease by 56 years	Capisonda et al. (2003), Guay-Woodford and Desmond (2003), Woon et al. (2015)

Note: Key characteristics of both diseases are listed by category.

Abbreviations: *DZIP1L*, DAZ interacting zinc finger protein 1 like; *PKHD1*, polycystic kidney and hepatic disease 1.

used to differentiate ARPKD from ADPKD at early stages. Milder ARPKD cases, which may be diagnosed later, are more similar to ADPKD including in cyst morphology (Adeva et al., 2006; Denamur et al., 2010; Sweeney & Avner, 2014). In contrast to ADPKD, where Tolvaptan treatment slows progress of the disease, there are no specific treatments for ARPKD patients and the standard-of-care approach for ARPKD is transplantation. Several studies have highlighted the risk of hepatobiliary disease, namely Caroli syndrome and related cholangitis, in causing morbidity and mortality in some post-renal transplant ARPKD patients (Büscher et al., 2014; Chapal et al., 2012; Davis et al., 2003; De Kerckhove et al., 2006). Therefore, 7% of such patients need a subsequent hepatic transplant (Guay-Woodford & Desmond, 2003). Combined kidney and liver transplant is considered highly beneficial to selected patients, although the opposite has also been reported (Büscher et al., 2015; Chandar et al., 2015; Mekahli et al., 2016; Telega et al., 2013).

ARPKD is almost always caused by variants in the polycystic kidney and hepatic disease 1 (*PKHD1*) gene (Onuchic et al., 2002; Lu et al., 2017). *PKHD1* contains 86 exons and is one of the largest genes in the human genome. The longest transcript, comprising 67 exons, encodes a 4074-residue protein named fibrocystin (FC), also known as polyductin. FC is a single-pass membrane protein of unknown function, although it has been implicated in early kidney development, and cell signalling, adhesion, proliferation, morphology and polarisation (Kim, Fu et al., 2008; Sweeney & Avner, 2006; Yang et al., 2007; Ziegler et al., 2020). ARPKD displays a high degree of allelic heterogeneity, with disease-causing variants found along the length of FC, with no obvious hotspots, providing little insight into the molecular pathogenesis of the disease (Bergmann et al., 2005; Gunay-Aygun et al., 2010). Genotype–phenotype relationships in ARPKD remain relatively poorly defined. In general, patients with two truncating *PKHD1* alleles die in the prenatal/neonatal period, whereas patients surviving beyond this possess at least one missense allele (Connaughton et al., 2019). That said, some missense changes appear to be just as deleterious as truncations (Bergmann et al., 2005; Gunay-Aygun et al., 2010), such as the c.107C > T (p.Thr36Met), the most frequent ARPKD variant with a prevalence of ~20% in all cases (Bergmann et al., 2004). However, a recent study demonstrated that pathogenic missense variants in different regions of the FC molecule were associated with distinct clinical outcomes (Burgmaier et al., 2021). Missense variants in residues 709–1837 were associated with less severe renal disease, whilst variants in residues 1838–2624 and 2625–4074 led to hepatic disease of decreased and increased severity respectively. Similar findings have been reported in other cohorts, but evidence remains limited (Furu et al., 2003; Qiu et al., 2020). A related gene, *PKHDL1*, encodes FC-L, a 4243-residue single-pass membrane protein with roles in adhesion, particularly in T lymphocytes (Hogan et al., 2003), but no link between *PKDHL1* and ARPKD has been established. Although the vast majority of ARPKD cases are caused by *PKHD1* variants, recent work identified a very small number of ARPKD cases caused by homozygous missense variants in the DAZ interacting zinc finger protein 1 like gene (Hertz et al., 2022; Lu et al., 2017), thus expanding the genetic landscape of the disease.

2 | FIBROCYSTIN STRUCTURE

FC is 4074-residue, single-pass membrane glycoprotein comprising a large N-terminal ectodomain (residues 19–3858) and a cytoplasmic C-terminal tail of 192 residues (Figure 1). There are no published experimentally determined structures of any part of the protein. Bioinformatic analyses predict that the ectodomain contains 12 immunoglobulin-like plexin-transcription factor (IPT)/IPT-like domains, a protective antigen 14 (PA14) domain, a sperm protein, enterokinase and agrin (SEA) domain and two regions of TMEM2 homology each with at least nine parallel beta-helix 1 (PbH1) repeats (He et al., 2006; Onuchic et al., 2002; Pei & Grishin, 2017; Rigden et al., 2004; Ward et al., 2002) (Table 2). The protein runs at a molecular weight of ~500 kDa on SDS-PAGE, reducing to 450 kDa (its predicted molecular weight) after PNGase treatment, showing that the ectodomain is heavily *N*-glycosylated (Bakeberg et al., 2011; Outeda et al., 2017). The cytoplasmic tail is predicted to be unstructured and contains putative ciliary targeting and nuclear localisation sequences (CTS and NLS) (Follit et al., 2010; Hiesberger et al., 2006), a polycystin-2 (PC2) interacting region (Kim, Li et al., 2008) and at least three PKA/PKG phosphorylation sites (Onuchic et al., 2002; Ward et al., 2002). The oligomeric state of the protein has not been determined. FC appears to be conserved through may eukaryotic clades, with putative orthologues in green algae, protists and other single-celled lineages (Pei & Grishin, 2017). Many species also encode the related FC-like protein, particularly apparent in more recent ancestors. In humans, FC-like protein shares highly similar domain organisation to its longer counterpart and is predicted to share a similar number of IPT domains and TMEM2 homology regions (Hogan et al., 2003; Pei & Grishin, 2017).

The FC ectodomain is predicted to contain a chain of at least 9 IPT domains, which possess an immunoglobulin-like fold. Other cell surface receptors in humans, such as plexins and RON tyrosine kinases, possess shorter chains of IPT domains that play roles in ligand

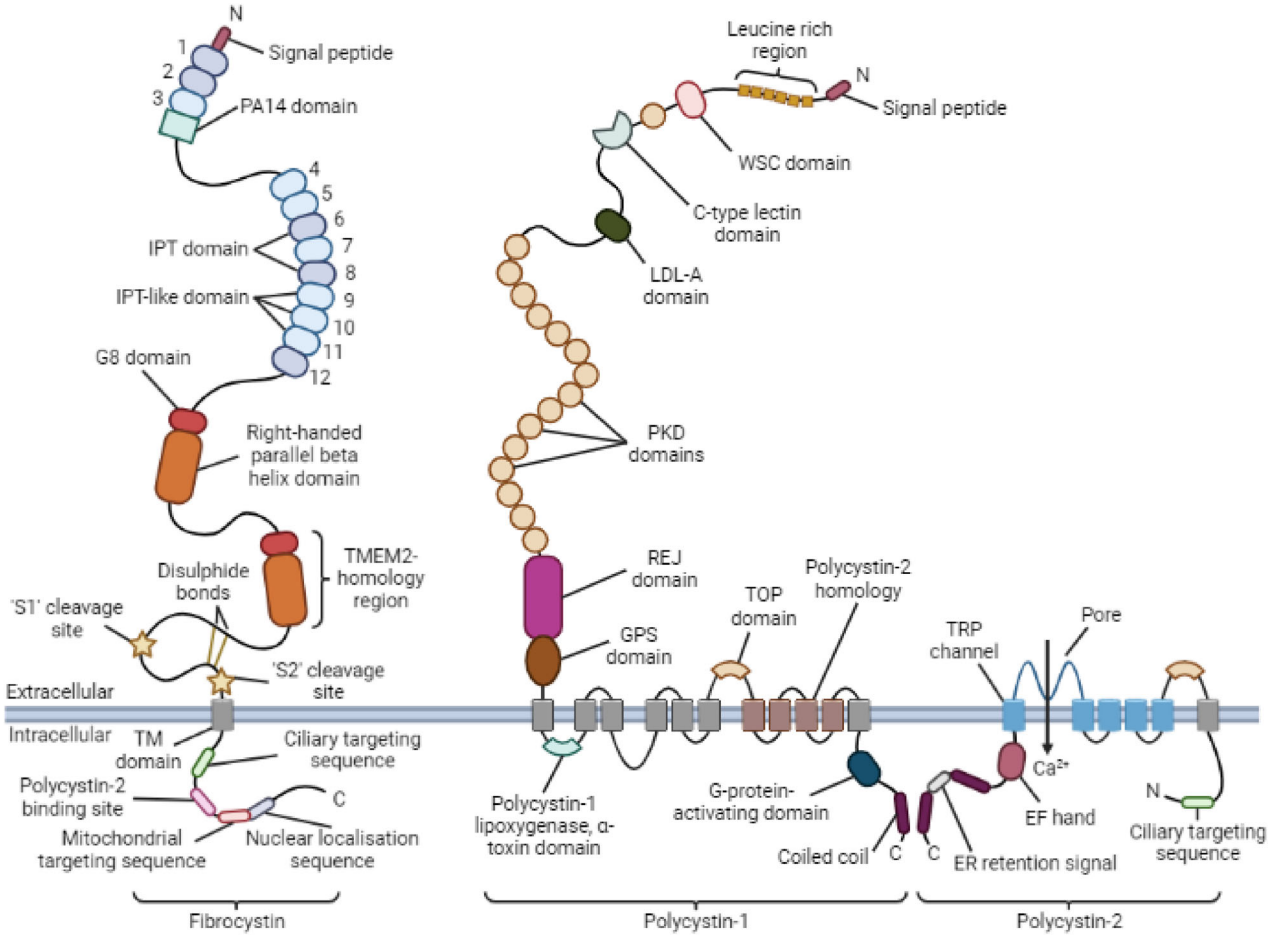

FIGURE 1 The PKD proteins: fibrocystin, polycystin-1 and polycystin-2. Schematics showing domain architecture and key features of fibrocystin, polycystin-1 and polycystin-2. Fibrocystin is a single-pass transmembrane protein which is found localised to the primary cilium. Polycystin-1 is an integral membrane protein which may modulate G protein coupled receptor (GPCR) signalling. Polycystin-2 is a tetrameric transient receptor potential (TRP) cation channel. Polycystin-1 and polycystin-2 form a heterotetrametric receptor–channel complex, with essential interactions between the polycystin-1 and -2 intracellular coiled coil domains (Newby et al., 2002; Su et al., 2018). ER, endoplasmic reticulum; IPT, immunoglobulin-like, plexins, transcription factors; LDL-A, low-density lipoprotein-A; PA14, protective antigen 14; PKD, polycystic kidney disease; REJ, receptor for egg jelly; TOP, tetragonal opening for polycystins; WSC, cell wall integrity and stress response component.

binding and dimerization (Basilico et al., 2014; Chao et al., 2012; Suzuki et al., 2016; Yotoko et al., 2019). In these proteins the presence of multiple IPT domains linked end-to-end imparts a curved shape to their ectodomains which, in some cases, may regulate dimerization and signalling (Figure 2a) (Basilico et al., 2014; Chao et al., 2012). In *Dictyostelium*, the TgrB1 and TgrC1 proteins mediate cell–cell adhesion through direct interactions between their IPT domain triplets (Chen et al., 2013). Recently, TgrB1 was shown to act as a receptor for TgrC1. These proteins enable coordination between individual cells via allorecognition, and binding has been shown to induce phosphorylation of the TgrB1 intracellular tail, triggering downstream signalling in control of differentiation (Hirose et al., 2015, 2017). It is therefore plausible that the IPT domains of FC could bind to as-yet to be determined ligands, modulate oligomerisation of the protein and mediate adhesive interactions, or some combination of these.

The PA14 domain is a globular, β-barrel domain found in all domains of life and is implicated in binding to carbohydrates (Rigden et al., 2004). Analysis with InterPro reveals only 5 human proteins other than FC and FC -like that possess a PA14 domain (UniProt IDs E9PHD9, Q6L9W6, Q6ZQZ4, Q76KP1 and Q8N9V0). Of these only two, the galactosyltransferases B4GALNT3 and B4GALNT4, have well characterised biological functions, mediating protein-specific transfer of *N*-acetylgalactosamine to *N*- and *O*-linked glycans in the Golgi (Fiete et al., 2012a, 2012b). The role of the PA14 domain in these processes is, however, unknown. In bacteria and fungi, PA14 domains are found in cell surface-exposed proteins that mediate cell

TABLE 2 Database-published predicted domains.

Position	Structural motif	Feature	Notes
258–355	IPT domain	IPT-PA14 region	PA14 could enable carbohydrate binding, IPT domain may act to stabilise or alter specificity
322–485	PA14 domain		
945–1001	IPT domain	IPT domain region	Extensive IPT domains may be important to target binding, possibly a peptide, and potentially enabling dimerisation. Comparable arrangement to plexin A1 may adopt a kinked shape
1018–1103	IPT domain		
1107–1193	IPT domain		
1195–1294	IPT domain		
1388–1482	IPT domain		
1486–1565	IPT domain		
1572–1659	IPT domain		
1932–2053	G8 domain	Helix-associated G8 and right-handed parallel beta helix/pectin lyase fold	Both G8 and PbH1 domains are carbohydrate binding, observed together in CEMIP and TMEM2. Relationship is not understood PbH1 domain thought to bind carbohydrates between a β-sheet and a loop, which may or may not be in fibrocystin
2226–2248	PbH1		
2249–2271	PbH1		
2292–2325	PbH1		
2326–2347	PbH1		
2409–2431	PbH1		
2469–2502	PbH1		
2740–2873	G8 domain	Helix-associated G8 and right-handed parallel beta helix/pectin lyase fold	Second G8-PbH1 region, following the previous in succession. One may possibly be the result of tandem duplication. Possible duplicate or extended binding site
3010–3032	PbH1		
3033–3055	PbH1		
3086–3108	PbH1		
3557–3717	SEA domain		Contains S1 cleavage site
3859–3881	TM region	Transmembrane	Single-pass hydrophobic helix
4033–4048	Low complexity	C-terminal tail	No defined structural regions detected

Note: Fibrocystin predicted domains were collated from InterPro (https://www.ebi.ac.uk/interpro/) and corroborated with SMART (http://smart.embl.de/). Positions come from InterPro annotation, excluding the final two, from SMART. The sperm protein, enterokinase and agrin (SEA) domain was reported in Pei and Grishin (Pei & Grishin, 2017).

Abbreviation: IPT, immunoglobulin-like plexin-transcription; PA14, protective antigen 14; PbH1, parallel beta-helix 1.

adhesion via direct, calcium-dependent carbohydrate binding (Figure 2b) (Irie et al., 2021; Shostak et al., 2014; Veelders et al., 2010: Zhang et al., 2021). This drives a number of important processes, for example yeast cell flocculation (a form of social behaviour), host epithelial cell adhesion by pathogenic fungi such as *Candida glabrata*, and bacterial cell adhesion to nutrient-rich substrates. In other cases, PA14 domains modify substrate specificity in bacterial and yeast carbohydrate processing enzymes. In β-glucosidase from the yeast *Kluyveromyces marxianus* (KmBglI), the PA14 domain is located at the active site entrance and restricts enzyme specificity to disaccharides by limiting access of longer substrates. Clostridium spp. Bgxa is a multifunctional β-glucosidase/β-xylosidase/α-arabinosidase with homology to KmBglI. In this protein, the PA14 domain is replaced by a flexible loop, which enables wider substrate specificity (Fiete et al., 2012b). Conversely, in the soil bacterium *Cellvibrio japonicus*, the PA14 domain of α-xylosidase from extends the active site, allowing binding to long, branched-chain oligosaccharides (Fiete et al., 2012a). The PA14 domain is conserved in the *Chamydomonas reinhardtii* FC homologue CHLRE_10g436800v5 (XP_042920129.1; residues 346–520). This suggests that the PA14 domain was present in the FC homolog from the last common ancestor of animals and plants and its conservation indicates that it performs an important function, presumably involving carbohydrate binding. Perhaps the FC PA14 domains bind to as-yet unidentified glycoprotein ligands at the cell surface, perhaps in conjunction with the surrounding IPT domains.

G8 domains are predicted to contain five repeated β-strand pairs and an α-helix and are named for their conserved glycine residues. Nine ARPKD missense variants have been identified in the FC G8 domains, including in the conserved glycine residues, demonstrating that these domains are crucial for function (He et al., 2006). G8 domains also occur in two hyaluronidases, CEMIP and TMEM2. In CEMIP, a secreted protein with roles in hearing loss and cancer, the G8 domain is linked to scaffolding, cell signalling and adhesion. The CEMIP G8 domain enables membrane targeting through ANXA1 binding, found to promote pathogenesis in rheumatoid

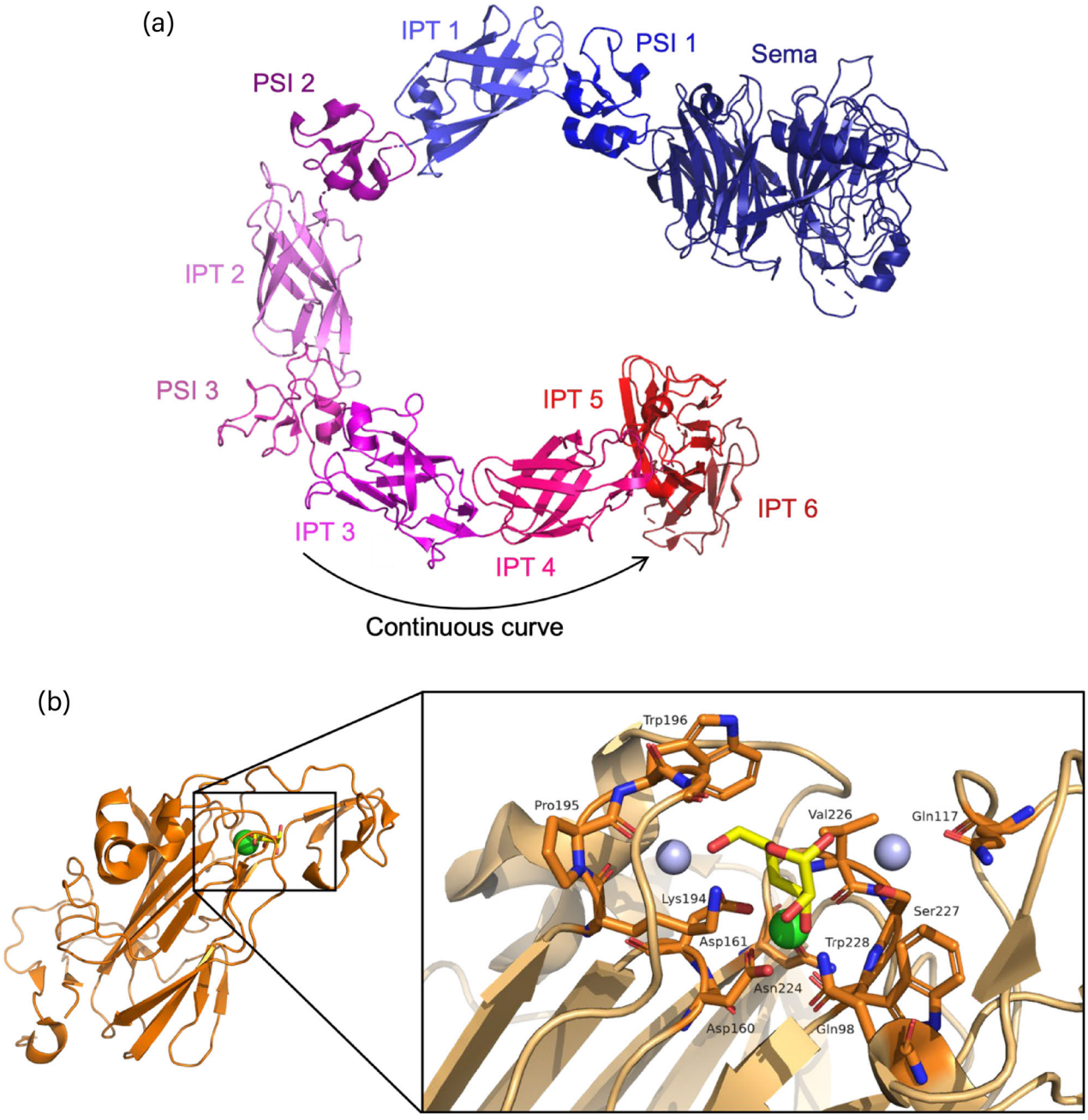

FIGURE 2 Structures of immunoglobulin-like plexin-transcription factor (IPT) and protective antigen 14 (PA14) domains. (a) The crystal structure of the plexin A1 ectodomain (PDB: 5L59) possess a curved chain of IPT domains (IPT3-5) (Kong et al., 2016). **(b)** The structure of the N-terminal (PA14) domain of *Saccharomyces cerevisiae* flocculin 1 (N-Flo1p; PDB: 4LHN) with bound mannose (ball-and-stick; carbon – yellow, oxygen – red) and a calcium ion (green sphere) in the carbohydrate binding site (Goossens et al., 2015). A close-up view of the binding site is shown, detailing the N-Flo1p residues (carbon – orange, oxygen – red and nitrogen blue) and bound water molecules (light blue spheres) that stabilise binding. *Source*: Generated in PyMOL (4.6.0).

arthritis (Zhang et al., 2021). The CEMIP G8 domain can also bind both plexin A2 and epidermal growth factor receptor (EGFR), promoting pro-survival EGFR signalling and antagonising pro-apoptotic plexin A2 signalling (Shostak et al., 2014). Perhaps the CEMIP G8 domain binds to the plexin A2 IPT domains, suggesting that the G8 and IPT domains of FC could also interact with each other, affecting FC conformation and/or dimerization. The TMEM2 ectodomain is structurally homologous to CEMIP, exhibits comparable activity and enables cellular adhesion to carbohydrates and integrins, but possesses a transmembrane domain before its G8 domain (Figure 3) (Irie et al., 2021). The extent of CEMIP and TMEM2 involvement in the catalysis of hyaluronan

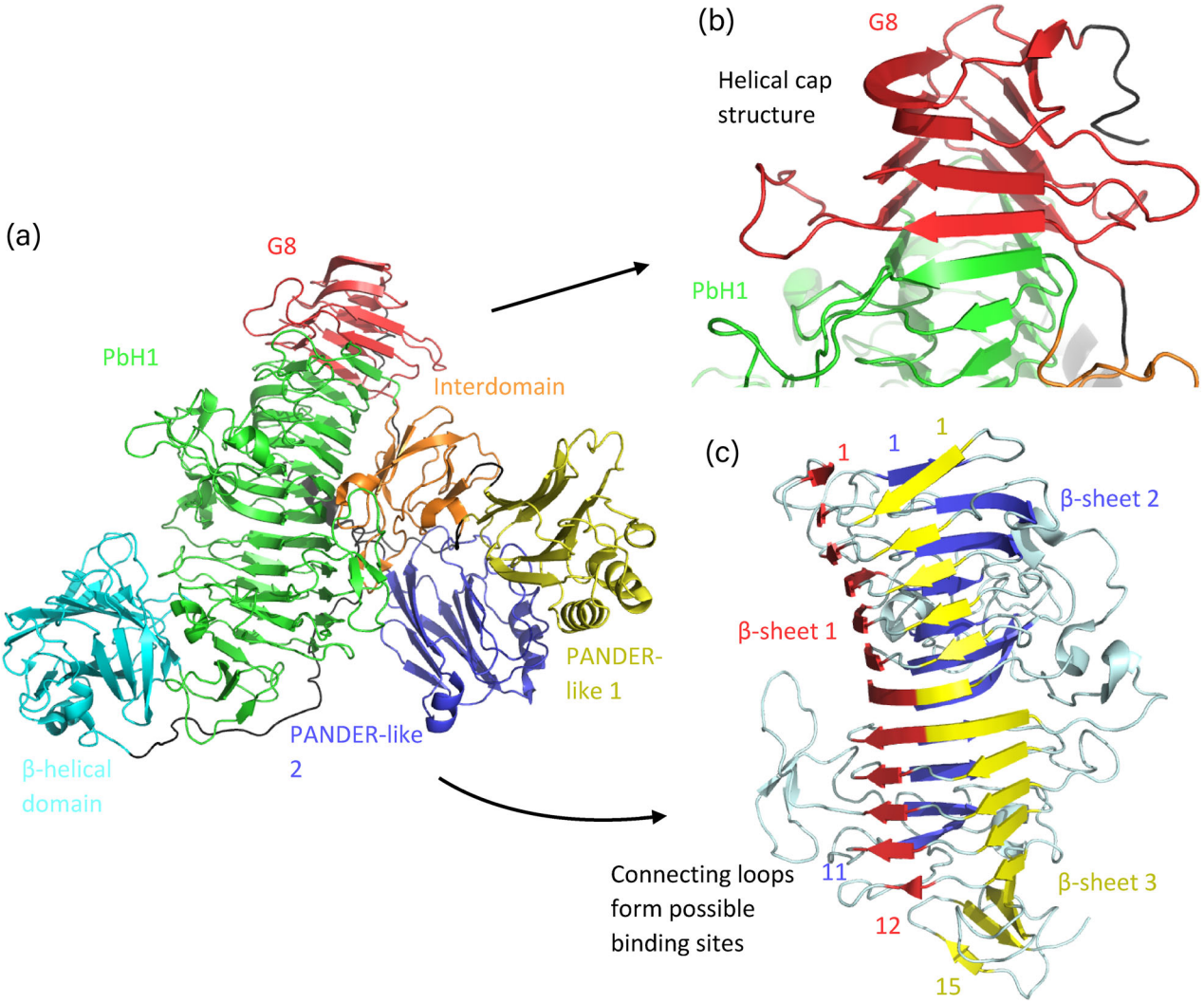

FIGURE 3 The crystal structure of the TMEM2 ectodomain. (a) Overview of the structure (PDB: 8C6I) with domains colour-coded and labelled (Niu et al., 2023). Fibrocystin is predicted to have two G8 domains, each followed by parallel β-helical repeats, possibly folding into a similar conformation as those of TMEM2. Part (b) shows a close-up of the G8 domain, which forms an N-terminal cap on the parallel beta-helix 1 (PbH1) domain. G8 loops may be linked to carbohydrate binding. (c) Structural details of the PbH1 domain. The PbH1 domain comprises three β-sheets (coloured differently) which form a right-handed helix. β-strands are numbered. β-sheet 3 (yellow) includes additional β-strands not from PbH1 repeats. Extended loops between repeats form binding pockets. These binding pockets lack much secondary structure and so may be undetected in previous predictions of fibrocystin structure.

depolymerisation is not yet proven, and both may primarily act as scaffolds (Spataro et al., 2022; Yamamoto et al., 2017; Yoshino et al., 2018).

The G8 domains of both CEMIP and TMEM2 are followed by PbH1 repeats, forming a predicted arrangement of a head with a β-helical trunk (Figure 3) (Spataro et al., 2022). Each PbH1 repeat includes three β-strands, capable of stacking to form a right-handed β-helix of three wound β-sheets. Disruption of the β-helical region with single missense variants was sufficient to abolish protein function in worms (Mayans et al., 1997). Based on homology, the β-helix of CEMIP is predicted to serve similarly. Although the G8 domain seems to support carbohydrate binding, the PbH1 domain apparently is more intimately involved in carbohydrate catalysis. The significance of this domain configuration is unclear, but its recurrence suggests importance to hyaluronidase and FC carbohydrate binding. Two of these regions are found in FC, possibly arising as a tandem duplication event (He et al., 2006). The consequence is undetermined, possibly imparting multiple binding sites. PbH1 domains are found in another family of enzymes, pectin and pectate lyases, where the substrate binding pocket is suspected to be nestled between one of the β-sheets and a loop, and not requiring a G8 domain (Mayans et al., 1997). Other PbH1 domain proteins are also carbohydrate-binding, including the viral P22 tailspike

protein, and most function without a G8 domain (Kajava et al., 2001; Miller et al., 1998; Wang et al., 2016).

3 | FIBROCYSTIN EXPRESSION AND LOCALISATION

FC appears to be the sole protein species produced from the *PKHD1* gene. Although alternatively spliced *PKHD1* transcripts have been observed by various methods, there is no evidence for these at the protein level (Bakeberg et al., 2011; Boddu et al., 2014; Onuchic et al., 2002; Outeda et al., 2017). FC expression is highest in the kidney, where it localises to the apical surfaces of the collecting duct, proximal convoluted tubule, and thick ascending limb of the loop of Henle (Outeda et al., 2017; Wang et al., 2007; Zhang et al., 2004). High expression is also seen in the ducts and islets of the pancreas, and the hepatic bile duct (Onuchic et al., 2002; Ward et al., 2003). In mice, expression in neural tube epithelial cells at begins at embryonic day 9.5, and in the ureteric bud and mesonephric tubules by day 11.5, with continued expression into expected regions during early nephron differentiation (Zhang et al., 2004).

Numerous studies have demonstrated that FC localises to primary cilia and basal bodies in cultured cells and in tissue (Bakeberg et al., 2011; Masyuk et al., 2003; Menezes et al., 2004; Wang et al., 2004, 2007; Ward et al., 2003; Wu et al., 2006; Zhang et al., 2004). Primary cilia are microtubule-based organelles that extend from the apical surface of most mammalian cells. They play diverse roles in chemo-, mechano- and photosensation, allowing us to see, hear, excrete and reproduce, and are essential for vertebrate developmental and growth factor signalling pathways, such as Shh, PDGF and Wnt. Ciliary dysfunction is associated with a broad spectrum inherited developmental disorders known as ciliopathies, characterised by retinal and renal degeneration, skeletal defects and CNS malformations (Reiter & Leroux, 2017). The ciliopathies nephronophthisis and Bardet–Biedl syndrome are renal cystic disorders that display phenotypic overlap with ARPKD (McConnachie et al., 2021). Primary cilia in *Pkhd1* knock-out (KO) mice display a range of morphological defects (Kim, Fu et al., 2008; Woollard et al., 2007). In the PCD rat model of ARPKD loss of FC results in malformed cilia in isolated intrahepatic bile duct cholangiocytes (Masyuk et al., 2003). These observations demonstrate that ARPKD is a ciliopathy.

4 | PROTEOLYTIC PROCESSING OF FIBROCYSTIN

A series of studies demonstrated that FC is proteolytic processed in a manner analogous to Notch. Notch receptors are single-pass transmembrane proteins that activate the Notch signalling pathway upon binding a ligand on the surface of another cell. They are synthesised as an immature, single polypeptide chain precursor that is post-translationally cleaved into the mature, disulphide-linked heterodimer by furin-like proteases en route through the secretory pathway (IS et al., 2006; Koulen et al., 2002; Wang et al., 2019). At the cell surface, ligand binding to the mature Notch receptor promotes juxtamembrane proteolytic cleavage of the Notch ectodomain by ADAM family proteases, followed by intramembrane cleavage of the transmembrane region by γ-secretases. These S2 and S3 cleavage events release the Notch ectodomain and intracellular domain (ICD); the latter transits to the nucleus and triggers a transcriptional response (Dalagiorgou et al., 2010; Fedeles et al., 2014; Lu et al., 1997; Pennekamp et al., 2002). Notch signalling is regulated by the Notch negative regulatory region (NRR). In the resting state of the mature receptor, the NRR occludes the S2 site, preventing ADAM protease cleavage. Ligand binding induces a conformational change in the NRR that exposes the S2 site, promoting downstream cleavage and signalling events.

Experiments with over-expressed, epitope-tagged FC constructs in cultured cells have demonstrated that FC is synthesised as a single polypeptide precursor (which we denote FC0). En route through the secretory pathway, the FC0 ectodomain is cleaved by an as-yet unidentified proprotein convertase enzyme (Figure 4). This likely occurs at a predicted proprotein convertase cleavage site between residues 3619 and 3620 (Kaimori et al., 2007). The N- and C-terminal cleavage fragments, which we denote FC1 and FC2, respectively, are linked into a heterodimer (denoted FC1/FC2) by inter-chain disulphide bonds, likely involving residues Cys^{3341} and Cys^{3346} on FC1 and Cys^{3622} and Cys^{3627} on FC2 (Kaimori et al., 2007). The ectodomain of the mature protein at the cell surface comprises the entire FC1 fragment (predicted to comprise residues 19-3619) and the N-terminal, extracellular region of FC2 upstream of the transmembrane helix (residues 3620–3850). Over-expressed, epitope-tagged FC underwent constitutive ectodomain shedding and intramembrane proteolysis, the latter releasing the FC ICD into the cytoplasm. These S2 and S3 cleavage events could be stimulated by a variety of agents that stimulate ADAM protease activity in a manner sensitive to pharmacological γ-secretase inhibition. Furthermore, the ICD fragment displayed NLS-dependent nuclear targeting (Douguet et al., 2019; Zhang et al., 2004). Western blot analyses of neonatal and adult kidneys from the FPC-HA mouse allowed direct detection of the FPC2 and ICD fragments in vivo by Western for the first time. However, the ICD was not detected in the nucleus in this system.

Immunofluorescence microscopy and EndoH digests showed that at least half of the endogenous protein resides

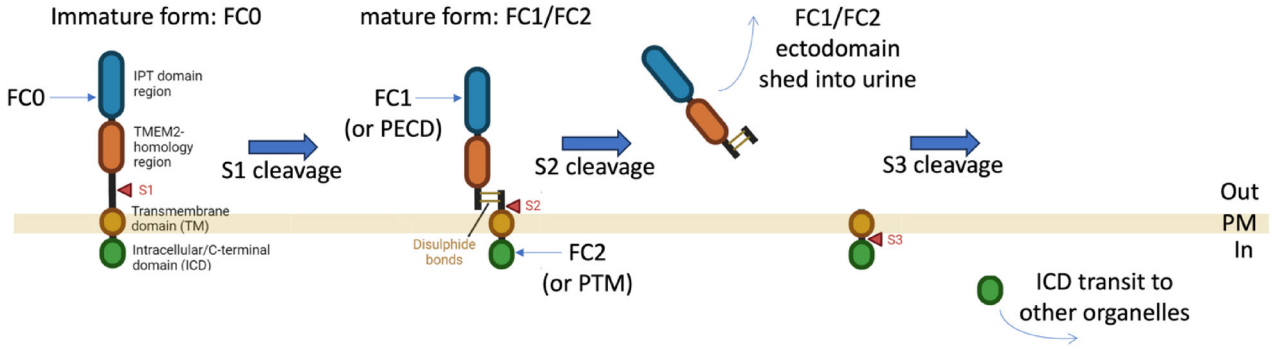

FIGURE 4 Notch-like proteolysis of fibrocystin. Cleavage of the immature fibrocystin precursor fibrocystin (FC0) at the proprotein convertase (S1) site produces the mature FC1/FC2 heterodimer. Note that FC1 and FC2 are also known as PECD and PTM, respectively, in other some studies (44,80)M. Juxtamembrane cleavage of F2 by ADAM metalloproteases sheds the F1/F2 ectodomain into the urine. S3 cleavage of the remaining membrane-bound protein by γ-secretases releases the intracellular domain (ICD) allowing it to transit to the nucleus and mitochondria.

in the uncleaved FC0 form in the endoplasmic reticulum in liver, kidney and pancreas in mice. Curiously, ciliary localisation of FC was not observed in epithelial tissue from the FC-HA knock-in mouse (Outeda et al., 2017). The reasons for this are not completely clear but may be due to levels of the protein being below the threshold for detection. FC and the ADPKD proteins, polycystin-1 (PC1) and PC2 are present at low levels in adult kidney tissue but are enriched in a fraction of urinary extracellular vesicles in humans and mice. Analysis of urinary extracellular vesicles (ELVs) from humans, and knock-in mice encoding N-terminal SV5-tagged or C-terminal HA-tagged FC, allowed endogenous FC to be characterised in vivo (Bakeberg et al., 2011; Outeda et al., 2017). Proteomics and Western blot analyses detected only FC1 and FC2 fragments in urinary ELVs, showing that ELV-associated FC exists as the FC1/FC2 heterodimer. In addition, FC1 was also identified in blots of urine samples from mice encoding N-terminally tagged FC (Bakeberg et al., 2011; Outeda et al., 2017). This species was not derived from ELVs (Bakeberg et al., 2011) and was presumably present as part of the FC1/FC2 heterodimer ectodomain shed from the epithelial cell surface by S3 cleavage.

The essence of the Notch paradigm is that exposure to a stimulus triggers receptor proteolysis, enabling regulated signalling via released components (Figure 5). In Notch, the S2 cleavage site resides within the SEA domain close to the transmembrane region. S2 proteolysis is regulated by the NRR immediately downstream, which blocks access to the S2 cleavage site in resting conditions. Mechanical force from endocytosis of the Notch receptor:ligand complex leads to exposure of the S2 cleavage site, allowing processing by alpha and gamma secretases and release of the ectodomain and intracellular fragments. Like Notch, FC has a large ectodomain but it has no known ligands. It is possible that mechanical stimuli, such as fluid flow, could induce conformational changes in the FC ectodomain, although FC does not appear to possess an equivalent of the Notch NRR. Thus, at present, it is unclear how regulated processing of FC could be allosterically triggered by a stimulus acting through the protein itself.

Previous work demonstrated that FC ectodomain shedding and release of the ICD can be enhanced by raising intracellular Ca^{2+}. This was demonstrated using a range of pharmacological treatments that raise intracellular Ca^{2+}. Calcium-triggered release of the ICD was found to be dependent on protein kinase C (PKC). PKC is regulated by intracellular Ca^{2+} release and diacylglycerol production, of which both are upregulated by the action of phospholipase C in the classical pathway (Nishizuka, 1986; Parker et al., 1989). PKC activation has been linked to the processing of amyloid precursor protein, mediated by sequentially acting α-secretase and γ-secretase. α-Secretases act constitutively but are upregulated by PKC; γ-secretases work downstream on α-secretase products, including Notch (Jurisch-Yaksi et al., 2013; MacLeod et al., 2015; O'Brien & Wong, 2011). The physiological triggers that could raise intracellular Ca^{2+} to promote FC cleavage are unclear but one possibility is flow-induced Ca^{2+} signalling through the primary cilium (Djenoune et al., 2023; Katoh et al., 2023; Piperi & Basdra, 2015; Praetorius & Spring, 2001, 2003).

Mature FC is released into urine in two forms. The full-length FC1/FC2 heterodimer is found in urinary ELVs. Proteomics and electron microscopic experiments indicated that FC-containing ELVs originate from multivesicular bodies (Bakeberg et al., 2011). Other work has also suggested that ELVs may bud off from bulb-like swellings on the primary cilium membrane of epithelial cells but whether such ELVs contain FC is unknown (Mohieldin et al., 2015; Zuo et al., 2019). The released vesicles are proposed to mediate 'urocrine signalling', where they are carried in the lumen to mediate a form of paracrine signalling by inserting into ciliary membranes. Immune-electron microscopy demonstrated that FC-containing ELVs bind to

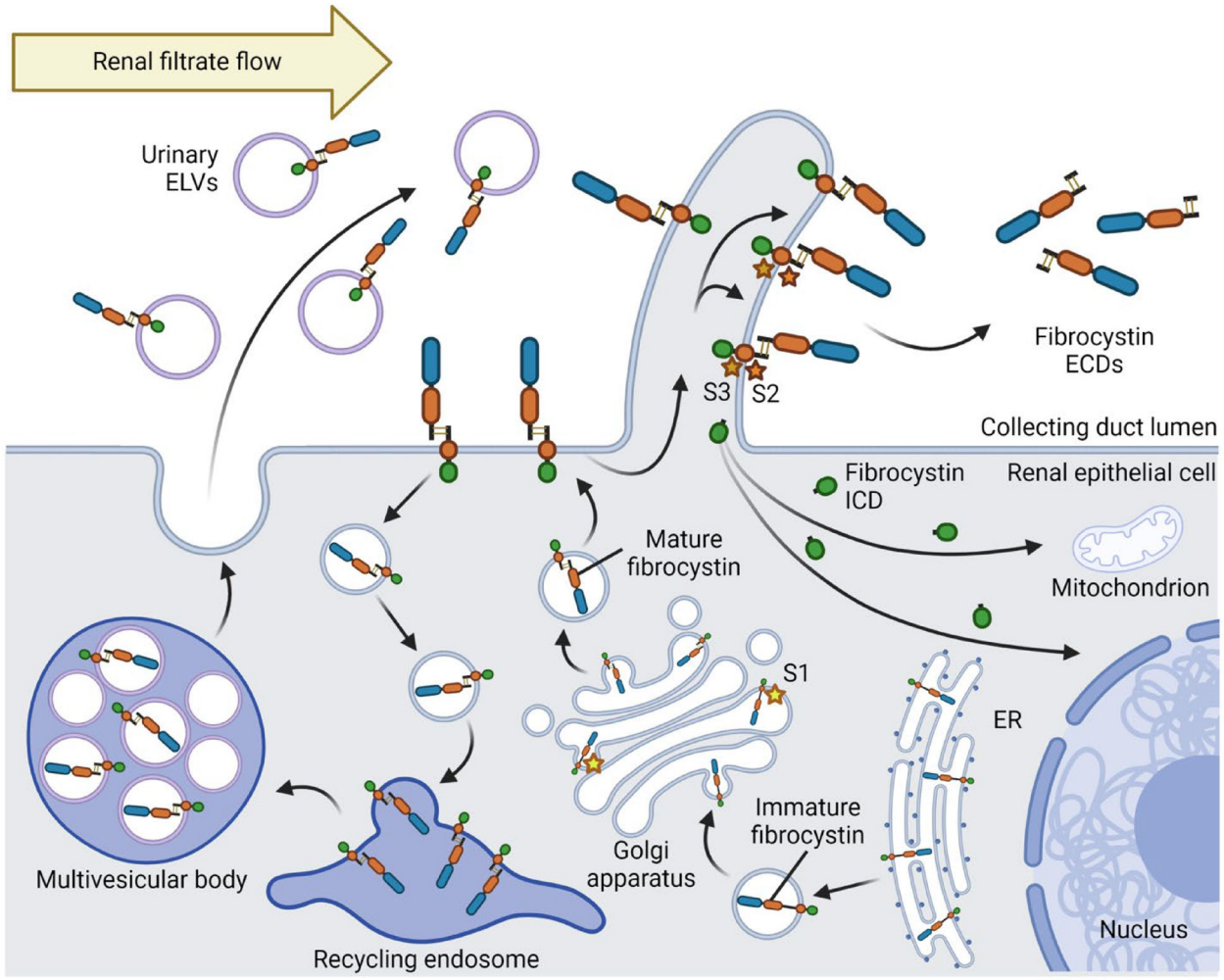

FIGURE 5 Summary of fibrocystin processing and trafficking based on (Kajava et al., 2001; Mayans et al., 1997; Mochizuki et al., 1996; Spataro et al., 2022) and (Bergmann, 2015). Fibrocystin is synthesised as a single polypeptide chain precursor fibrocystin (FC0) at the ER and is transported through the secretory pathway. S1 cleavage by proprotein convertase enzymes in the Golgi apparatus generates the mature FC1:FC2 heterodimer that is delivered to the cell surface. The FC1/FC2 heterodimer may be laterally trafficked into the primary cilium membrane, where S2 and S3 cleavage shed the ectodomain into the lumen and release the intracellular fragment which transits to the nucleus and mitochondria. S2 and S3 cleavage may be promoted by an increase in intracellular Ca^{2+} induced by deflection of the primary cilium by fluid flow. Other FC1/FC2 molecules in the plasma membrane may be re-internalised from the plasma membrane and trafficked to the recycling endosome, where they are packaged into extracellular vesicles to form multivesicular bodies (MVBs). Fusion of MVBs with the plasma membrane releases FC1/FC2 containing extracellular vesicles (ELVs) into the collecting duct lumen. FC1/FC2 containing ELVs may also be released from primary cilia.

bile duct cilia (a tractable model system for quasi-in vivo studies). FC was subsumed into and cleared from ciliary membrane following ELV binding. Intriguingly, in ARPKD patients or in *Pkhd1* KO mice, ELVs displayed enhanced clustering onto bile duct cilia, with no fusion of ELV and ciliary membranes observed. This suggests that FC could mediate a post-attachment step of ELV fusion with the ciliary membrane (Bakeberg et al., 2011; Hogan et al., 2009). The function of this pathway is not yet known, although it has been theorised to relate to cellular polarity (Patel, 2011). The ectodomain of the FC1/FC2 heterodimer is released into urine as a soluble protein. This species presumably acts as a urinary signalling molecule. However, the fate of this species and its role in renal development and function remain to be elucidated.

In the Notch pathway, the cytoplasmic fragment generated by intramembrane proteolysis transits to the nucleus and initiates a transcriptional response. Hiesberger et al. (2006) observed the ICD of endogenous FC in the nuclear fraction of cultured human IMCD3 cells by Western blot using an antibody against the C-terminus of the protein. An antibody against the C-terminal region of the protein also labelled the nuclei in sections from mouse renal columnar epithelial cells. When overexpressed in cultured

cells, tagged ICD constructs displayed NLS-dependent nuclear targeting as judged by fluorescence microscopy and in one study co-localised with nucleoli (Hiesberger et al., 2006). The functional relevance of these observations remains unclear. However, knock-in mice lacking *Pkhd1* exon 67 (encoding most of the ICD including the NLS and putative PC2 binding site) did not express a kidney or liver phenotype and appeared healthy (Outeda et al., 2017). The ICD also contains a putative PC2 binding region, but in kidney lysates from FC-HA knock-in mice the HA-tagged FC did not immunoprecipitate PC2. It therefore appears that the FC ICD is dispensable in mice. However, human FC shares relatively low sequence identity with the mouse over the ICD, and *Pkhd1* KO mice develop a kidney phenotype distinct from that found in human ARPKD patients (Gallagher et al., 2008; Moser et al., 2005; Nagasawa et al., 2002). In ARPKD patients, the transcription factor STAT3 is upregulated in cyst-lining renal epithelial cells, and the cytoplasmic region of FC was shown to bind STAT3 directly and suppress SRC-activated STAT3 signalling in cultured human cells (Dafinger et al., 2020). In addition, overexpression of an ICD construct in cultured human cells was shown to antagonise full-length FC-mediated inhibition of the mTOR pathway, promoting cystogenesis in vitro, suggesting a self-regulatory mechanism (Wang et al., 2014). Thus, the role of the FC ICD within a Notch-like signalling pathway remains to be demonstrated, but it may play roles in human health and disease not well modelled in mice.

Further to its potential role in the nucleus, recent work also implicates the ICD in mitochondrial function and energy metabolism. Gene-edited cultured HEK-293 cells carrying clinically relevant *PKHD1* truncating variants displayed aberrant mitochondrial morphology and increased oxygen consumption and extracellular acidification rates (Chumley et al., 2019). A very recent study identified a shorter ICD fragment (ICD_{15}) that localises to mitochondria via a cryptic mitochondrial targeting sequence (MTS) that overlaps partially with the NLS (Walker et al., 2023). Electron microscopy on renal tubule cells in kidneys from *Pkhd1* KO mice revealed mitochondria with significantly decreased surface area, increased roundness and swollen cristae, suggesting a change to the inner mitochondrial membrane. Although mice homozygous for *Pkhd1* exon 67 deletion appear healthy (Outeda et al., 2017), this deletion (which removes the MTS) enhanced renal cystogenesis in a *Pkd1*-defective background (Walker et al., 2023). The FC C-terminal domain may thus play complex roles in both the nucleus and the mitochondria.

5 | THE POLYCYSTIN COMPLEX (PCC)

In contrast to ARPKD, ADPKD is caused by variants of the *PKD1* and *PKD2* genes, which encode the PC1 and PC2 proteins (Mochizuki et al., 1996; Rossetti et al., 2007; The European Polycystic Kidney Disease Consortium, 1994). PC1 and PC2 form a Ca^{2+} permeable receptor-channel complex in the ciliary membrane (Figure 1) (Newby et al., 2002, Wang et al., 2019). PC2 belongs to the transient receptor potential family and forms a tetrameric ion channel (IS et al., 2006; Koulen et al., 2002). Binding to PC1 allows G-protein-mediated modulation of Ca^{2+} flow and regulatory signalling (Dalagiorgou et al., 2010; Fedeles et al., 2014). The PC1/2 complex is thought to function in Ca^{2+} signalling and ciliary mechanosensation and has been implicated in the development and regulation of cell–cell adhesion (Douguet et al., 2019; Forman et al., 2005; Huan & van, 1999; Kim et al., 2016; Lu et al., 1997; Pennekamp et al., 2002). Interactions at the protein and genetic level, protein co-localisation to cilia and ELVs, and phenotypic overlap between ARPKD and ADPKD, have led to the proposal that FC may function in complex with PC1/2 to form the putative polycystin complex (Liu et al., 2018).

PC2 and FC have been shown to co-localise and co-immunoprecipitate in human and mouse cell-lines and kidney tissue samples (Wang et al., 2007). This interaction was shown to involve a region of the FC ICD and the PC2 N-terminus (Kim, Fu et al., 2008; Kim, Li et al., 2008). Furthermore, antibody-mediated blockade of the FC ectodomain could reduce Ca^{2+} flux in response to sheer force, suggesting that the FC ectodomain can modulate PC2 function (Wang et al., 2007). At the genetic level, studies in mice showed that *Pkhd1* variants exacerbated renal cystic disease caused by *Pkd2* variants (Kim, Fu et al., 2008; Kim, Li et al., 2008). Similarly, immunogold electron microscopy studies showed that PC1, PC2 and FC co-localise in ELVs. Nevertheless, a direct interaction between FC and PC1/PC2 has not been demonstrated. This interaction may be bridged by heterotrimeric kinesin-2 (Wu et al., 2006). Heterotrimeric kinesin-2 drives anterograde intraflagellar transport of IFT-B trains and consists of the KIF3A-KIF3B-KAP3 complex (Scholey, 2013; Verhey et al., 2011). Yeast two-hybrid assays demonstrated a direct interaction between PC2 and both the KIF3A and KIF3B subunits, whereas the FC ICD bound directly to KIF3B. The endogenous proteins were found to coimmunoprecipitate from lysates from human and mouse tissues demonstrating that the proteins interact in cells. Furthermore, using an in vitro electrophysiological assay, FC and kinesin-2 were shown to synergistically modulate the conductance states of PC2 in an artificial membrane bilayer (Wu et al., 2006).

Primary cilia induce Ca^{2+} transients in response to fluid flow, detecting direction and determining left-right asymmetry; PC2 appears to be essential in this process (Djenoune et al., 2023; Katoh et al., 2023). Thus, the proper regulation of Ca^{2+} flux may have an impact on cellular polarity and morphology. Supporting shared FC-PC2

pathways, studies in mouse models have highlighted a synergistic relationship between knockout of *Pkhd1* and *Pkd1*, linking ARPKD and ADPKD combining genotypes worsened disease phenotype through a common molecular pathway (Garcia-Gonzalez et al., 2007; Olson et al., 2019). Impaired Ca^{2+} signalling is also associated with cystogenic proliferation. In healthy kidney cells, cAMP acts to reduce proliferation. Reduced intracellular Ca^{2+}, as seen in the cystic cells of ARPKD patients, induces a switch to a cAMP-stimulated proliferative phenotype. Enhanced proliferative B-Raf/MEK/ERK signalling triggered by cAMP can be reversed in vitro by treatment with a Ca^{2+} channel activator (Yamaguchi et al., 2006). FC may support normal PC2 activity, enabling appropriate intracellular signalling in response to ciliary shear forces.

6 | ROLES IN ADHESION

ARPKD patients present with renal tubule morphogenesis defects suggesting that loss of FC function negatively affects renal development, with evidence pointing towards an essential role for FC in cellular adhesion and motility. FC knock-down in IMCD3 cells induced defects in tubule formation and ciliogenesis (Mai et al., 2005). Fluorescence microscopy showed that the cell–cell junction proteins *E*-cadherin and ZO-1 did not localise to cell–cell contacts and displayed a diffuse cytoplasmic distribution. Adhesion assays showed impaired integrin-dependent adhesion. Cell survival and polarity, both modulated by adhesive signalling, were also altered by FC knock-down. ERK and FAK signalling was dysregulated, possibly contributing to irregular tubulomorphogenesis (Mai et al., 2005). FAK is a regulator of the focal adhesion complex. Focal adhesions are important to cell adhesion and migration and directly impact on morphogenesis and adhesion signalling pathways (Shemesh et al., 2005; Wehrle-Haller, 2012). Therefore, the marked decrease in FAK stimulation may have disrupted cell–cell contacts and affected the associated pathways. In support of this, other studies promote FC loss to increase cell detachment and migration. FC knockdown in MDCK cells caused increased invasion in collagen matrices and reductions in contractility, cell–cell adhesion and cell–matrix adhesion (Puder et al., 2019). FC depletion in MDCK cells has also been shown to drastically reduce the formation of spheroids and lead to unusual cell spreading and reduced numbers of focal adhesions (Davis et al., 2003). These morphological defects appear to arise from altered adhesion signalling, supported by the finding that treatment with blebbistatin to reduce contractile forces could restore cellular polarity, overcoming irregularities in cell surface adhesion molecules (Davis et al., 2003).

In contrast, another study found that loss of FC led to the opposite phenotype, that is increased adhesion and reduced motility (Israeli et al., 2010). Abnormal FAK phosphorylation patterns were observed in ARPKD patient cystic epithelial cells and kidney samples compared to healthy controls, with inhibitory sites being phosphorylated following in vitro adhesion to collagen, apparently resulting from increased SRC activation. Assays of cellular spreading on collagen showed ARPKD cells to have increased attachment and reduced migration. In IMCD3 cells, FC was found to interact with paxillin, the scaffold component of the focal adhesion complex, suggesting a role for FC in the function of focal adhesion complexes (Israeli et al., 2010).

7 | IMMUNE SYSTEM INVOLVEMENT IN CHF TRIGGERED BY FIBROCYSTIN DEFICIENCY

FC may influence cytokine release, and this may play a role in CHF. Cholangiocytes from *Pkhd1*-defective $Pkhd1^{\Delta 4/\Delta 4}$ mice were shown to secrete chemokines (Locatelli et al., 2016). This stimulated recruitment of macrophages that secreted pro-inflammatory cytokines, causing cholangiocytes to upregulate pro-fibrogenic signalling. Macrophage depletion resulted in a significant decrease in disease manifestations. FC-deficiency in cholangiocyte-like induced pluripotent stem cells increased interleukin-8 secretion, consistent with patient liver samples (Tsunoda et al., 2019). MAPK signalling was activated by FC loss, promoting interleukin-8 release and autocrine-mediated cystogenic proliferation. These studies highlight the role of the immune system in liver disease caused by FC deficiency.

8 | CONCLUSION

FC is a very large membrane protein located at the cell surface but the molecular details of its function remain obscure. The protein may be a cell surface receptor but the signals it detects and how these are relayed to the rest of the cell are unknown. The FC ectodomain possesses multiple domains that are implicated in protein:carbohydrate interactions suggesting that the protein may bind to as-yet undetermined carbohydrate and/or glycoprotein ligands. The long chains of IPT domains may conformationally regulate self-association of the protein and/or binding to other ligands by analogy to other smaller receptors IPT domain-rich ectodomains. Proteolytic processing of FC shares parallels with Notch receptors. How do these proteolysis events relate to/regulate signalling remains a mystery. FC localises to the primary cilia of renal and hepatic epithelial cells and is released into the extracellular

medium on extracellular vesicles, whilst the ectodomain is shed by proteolysis. The role(s) of these species remains unclear but given that FC is likely to be one of the largest proteins on the surface of primary cilia and urinary ELVs and that urinary ELVs bind to cilia in a FC-dependent manner, it seems likely that the protein will be involved in these interactions. Around two thirds of ARPKD births survive the neonatal period and display a highly variable clinical course, providing an untapped therapeutic window that could be exploited to improve the lives of many ARPKD patients. Further progress in understanding the molecular structure and function of FC will be essential to achieving this goal.

AUTHOR CONTRIBUTIONS

Study conception and design: Travis A K Bannell and Joseph J B Cockburn. *Analysis and interpretation of results*: Travis A K Bannell and Joseph J B Cockburn. *Draft manuscript preparation*: Travis A K Bannell and Joseph J B Cockburn. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ORCID

Joseph J B Cockburn https://orcid.org/0000-0003-4730-1436

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