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ORIGINAL ARTICLE

Impaired regulation of PMCA activity by defective CFTR expression promotes epithelial cell damage in alcoholic pancreatitis and hepatitis

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Abstract

Alcoholic pancreatitis and hepatitis are frequent, potentially lethal diseases with limited treatment options. Our previous study reported that the expression of CFTR Cl^- channel is impaired by ethanol in pancreatic ductal cells leading to more severe alcohol-induced pancreatitis. In addition to determining epithelial ion secretion, CFTR has multiple interactions with other proteins, which may influence intracellular Ca^{2+} signaling. Thus, we aimed to investigate the impact of ethanol-mediated CFTR damage on intracellular Ca^{2+} homeostasis in pancreatic ductal epithelial cells and cholangiocytes. Human and mouse pancreas and liver samples and organoids were used to study ion secretion, intracellular signaling, protein expression and interaction. The effect of PMCA4 inhibition was analyzed in a mouse model of alcohol-induced pancreatitis. The decreased CFTR expression impaired PMCA function and resulted in sustained intracellular Ca^{2+} elevation in ethanol-treated and mouse and human pancreatic organoids. Liver samples derived from alcoholic hepatitis patients and ethanol-treated mouse liver organoids showed decreased CFTR expression and function, and impaired PMCA4 activity. PMCA4 co-localizes and physically interacts with CFTR on the apical membrane of polarized epithelial cells, where CFTR-dependent calmodulin recruitment determines PMCA4 activity. The sustained intracellular Ca^{2+} elevation in the absence of CFTR inhibited mitochondrial function and was accompanied with increased apoptosis in pancreatic epithelial cells and PMCA4 inhibition increased the severity of alcohol-induced AP in mice. Our results suggest that improving Ca^{2+} extrusion in epithelial cells may be a potential novel therapeutic approach to protect the exocrine pancreatic function in alcoholic pancreatitis and prevent the development of cholestasis in alcoholic hepatitis.

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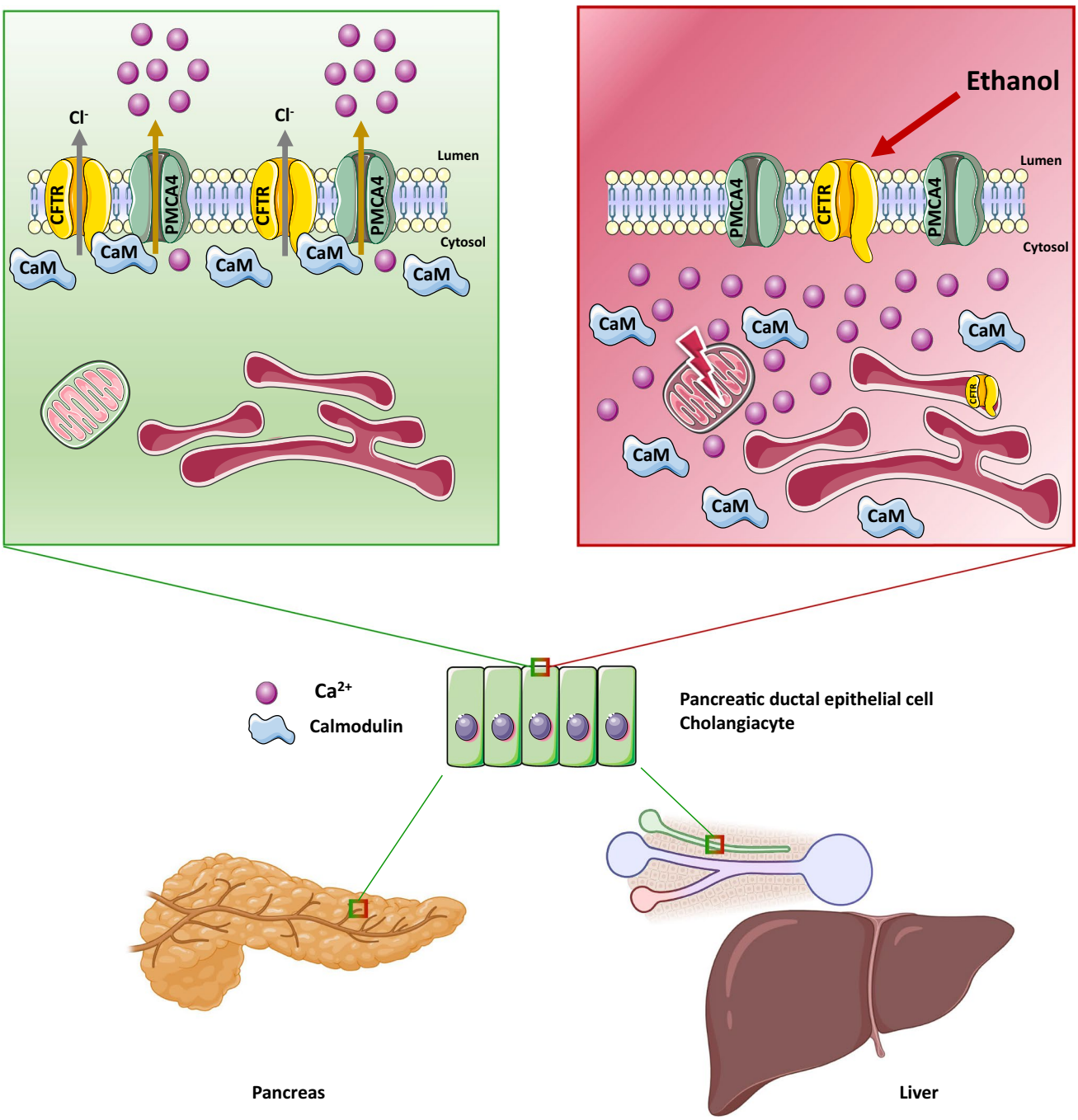
Graphical abstract



Keywords Alcoholic hepatitis · Alcoholic pancreatitis · CFTR · Ca^{2+} signaling · Epithelial ion secretion

Abbreviations

AH	Alcoholic hepatitis
AP	Acute pancreatitis
CF	Cystic fibrosis
CFLD	CF-associated liver disease
CFTR	Cystic fibrosis transmembrane conductance regulator
CPA	Cyclopiazonic-acid
DMSO	Dimethyl sulfoxide
EPI	Exocrine pancreatic insufficiency

ER	Endoplasmic reticulum
EtOH	Ethanol
FAEE	Fatty acid ethyl ester
HPO	Human pancreatic organoids
IP3R	Inositol triphosphate receptor
iPSC	Induced pluripotent stem cells
ITGB1	Integrin beta-1
KRT19	Cytokeratin 19
MLO	Mouse liver organoids
MPO	Mouse pancreatic organoids
PA	Palmitic acid
PMCA	Plasma membrane calcium pump
POA	Palmitoleic acid
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule 1
ΔΨm	Mitochondrial membrane potential

Introduction

Annually, 3 million deaths result from excessive alcohol consumption worldwide representing 5.3% of all deaths, whereas in the age group 20–39 years approximately 13.5% of the total deaths are attributed to alcohol [1]. Among alcohol-related disorders the diseases of the liver and pancreas emerge due to their therapeutic challenges and socioeconomic burden [1, 2]. Alcohol induced acute pancreatitis (AP) is one of the most frequent form of AP associated with high mortality in severe cases [3]. Our group evidenced previously that ethanol and fatty acid-mediated reductions of cystic fibrosis transmembrane conductance regulator (CFTR) expression and activity in pancreatic ductal cells cause decreased HCO_3^- secretion and increased severity of alcohol-induced AP [4]. We also showed that ethanol and fatty acids impair CFTR folding and reduce the plasma membrane stability of the protein. Whereas alcoholic hepatitis (AH) is a potentially lethal complication of alcoholic liver disease, which has been attributed to hepatocellular damage in the past [5]. Recent studies showed that cholestatic liver injury can be involved in the pathogenesis of AH, moreover, impaired secretion by cholangiocytes, or cholestasis, results in a worse outcome [6]. Takeuchi et al. [7] highlighted that integrin beta-1 (ITGB1)-mediated binding of neutrophils to cholangiocytes in AH results in the development of cholestasis. It is well established that cholangiocyte secretion largely depends on the proper function of the apically expressed CFTR [8]. Therefore, considering that the alcohol-mediated effect on CFTR expression has been observed in other organs, such as the sweat glands [4], it is tempting to speculate that impaired CFTR expression might contribute to AH-related cholestasis.

In general, lack of ion conductance and impaired fluid secretion, ultimately leading to morphological changes and atrophy of the organ, is considered central to the pathogenesis of CFTR-related disorders. However, a more complex, integrative role of CFTR was recently suggested in the regulation of intracellular signaling events, which are potentially involved in the development of CFTR-related cell- and tissue damage. For example, cystic fibrosis (CF)-associated increased IP_3R-dependent Ca^{2+} release [9], elevated activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, and enhanced mitochondrial Ca^{2+} uptake paralleled with decreased Ca^{2+} ATPase (PMCA) function [10] were described in cultured airway epithelial cells. Notably, alterations of intracellular Ca^{2+} signaling—such as sustained intracellular Ca^{2+} overload mediated by impaired SERCA- and PMCA activity—in pancreatic acinar and ductal cells is a well-established hallmark of AP [11]. Recently, Bozoky et al. [12] showed that calmodulin interacts with CFTR in the alternative binding conformation through independent binding of the two calmodulin lobes to two separate sequences allowing it to bridge larger distances. The authors suggested that calmodulin binding by CFTR may allow CFTR to recruit calmodulin and subsequently determine the activity of other calmodulin-regulated proteins, such as proteins involved in the regulation of the intracellular Ca^{2+} homeostasis. However, if and how these subcellular changes impact gastrointestinal tract epithelial cell function in alcoholic pancreatitis and hepatitis is currently unknown.

Thus, we aimed to investigate the effect of ethanol on CFTR activity and expression in cholangiocytes and analyze the impact of ethanol-mediated CFTR damage on intracellular Ca^{2+} homeostasis in pancreatic ductal cells and cholangiocytes in multiple independent model systems. In this study, we identified a novel regulatory interaction based on the apical recruitment of calmodulin by CFTR determining the activity of PMCA4 and intracellular Ca^{2+} extrusion in polarized epithelial cells. Thus, we propose that the prevention of the intracellular Ca^{2+} overload with improving PMCA activity might represent a novel potential drug target in alcoholic pancreatitis and -hepatitis and in CFTR-related diseases, such as CFLD, CF-related pancreatic disease.

Materials and methods

All materials, antibodies, primers, and solutions used in the study are listed in Supplementary Tables 1–8. Detailed protocols and descriptions of the applied methods are provided in the Supplementary Methods.

Cell lines and animals

HeLa cells were cultured according to the manufacturer's protocol [13]. CFPAC-1 cells were a generous gift of Michael Gray and were grown supplemented as described [14]. *Cftr* KO mice were originally generated by Ratcliff et al. [15]. Details of the animals used in the study are described in Supplementary Methods.

Isolation of pancreatic ductal fragments and acinar cells

Pancreatic ductal fragments were isolated as described earlier [4] under stereomicroscope. For pancreatic acinar cell isolation, the tissue was injected with type 4 collagenase and vigorously shaken followed by centrifugation after which the pellet was resuspended in Media 199 [16].

Mouse and human organoid cultures

Mouse pancreatic ductal and liver organoids were generated as previously described with modifications [17, 18]. Human pancreatic tissue samples were collected from transplantation donors [19]. The composition of different media is listed in Supplementary tables 2–5.

Generation of human CF-specific induced pluripotent stem cells

Reprogramming of keratinocytes isolated from plucked hair of a CF patient or a healthy donor was performed to generate CF-specific and control induced pluripotent stem cells as previously described [20]. The CF patient harbored the compound heterozygous mutations p.F508del and p.L1258Ffs*7.

Constructs and transfection

HeLa cells were transfected with plasmids coding EGFP-hPMCA4b, mCherry-CFTR-3xHA, and mCherry-CFTR-3xHA(S768A) using Lipofectamine2000 as described earlier [13]. Site-directed mutagenesis using Q5 Site-Directed Mutagenesis Kit was carried out according to the manufacturer's protocol. For *Cftr* silencing ducts were transfected with 50 nM siCFTR or siGLO-Green transfection indicator in Opti-MEM.

Fluorescent microscopy

$[Ca^{2+}]_I$, intracellular pH, and Cl^- level were measured with Fura-2-AM, BCECF-AM, or MQAE, respectively, as described earlier [17]. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were followed with tetramethylrhodamine-methyl ester (TMRM) [4]. Ducts, acini, or organoids were mounted on an Olympus IX71 fluorescent microscope equipped with an MT-20 illumination system. Filter sets for Fura-2, BCECF, and MQAE were described previously [17]. The signal was captured by a Hamamatsu ORCA-ER CCD camera through a 20× oil immersion objective (Olympus; NA: 0.8) with a temporal resolution of 1 s.

Gene expression analysis

Total RNA was isolated from whole pancreatic tissue, isolated ductal fragments, and pancreatic or liver organoids [17]. Relative gene expression analysis was performed by $\Delta\Delta Cq$ technique. RNA sequencing was carried out by an Illumina NextSeq 500 instrument on mouse and human pancreatic ductal organoids. The pattern of gene expression was determined by TPM (transcript/million) values.

Immunofluorescent- and immunohistochemical labeling and Duolink® proximity ligation assay

Isolated pancreatic ducts or organoids were frozen, sectioned, and labeled for immunofluorescent microscopy as previously described [4, 17]. Cell lines were grown on cover glass and fixed without sectioning. Applied antibodies are listed in Supplementary Tables 6–7. Immunohistochemical labeling of CFTR was carried out with a Leica Bond-MAX Fully Automated IHC and ISH Staining System. Line profile analysis was performed with FiJi (NIH-ImageJ). Duolink® assay was performed on a humidified chamber after antigen retrieval according to the manufacturer's protocol. Images were captured with a Zeiss LSM880 confocal microscope using a 40× oil immersion objective (Zeiss, NA: 1.4).

Direct stochastic optical reconstruction microscopy (dSTORM)

HeLa cells grown on cover glass were co-transfected with different plasmids. 2D primary human pancreatic ductal cell cultures were generated by digesting and plating human pancreatic ductal organoids on cover glass. For imaging, cover glasses were placed on cavity slides filled with blinking buffer and sealed with two-component adhesive. dSTORM images were captured by Nanoimager S (Oxford Nanoimaging ONI Ltd.). Cluster analysis of dSTORM images were evaluated by CODI (Oxford Nanoimaging ONI Ltd.).

Transmission electron microscopy

Pancreatic tissue was fixed in glutaraldehyde and dextran [21], embedded into Embed812, and ultrathin sections were

cut with a Leica ultramicrotome. Images were captured with a Jeol 1400 plus electron microscope at 12000× magnification. The volume fraction of the cells and mitochondria were calculated.

Ketone body measurement

Ketone Body production measured in blood serum samples of WT and CFTR KO mice with the Ketone Body Assay Kit (Abcam, ab272541) according to the manufacturer's protocol. Acetoacetic acid (AcAc) and 3-hydroxybutyric acid (BOH) levels were measured and total ketone body (TKB) concentration was calculated.

Cell viability assay

Mouse pancreatic ductal organoids were grown in Cultrex Ultimatrix until passage number 3. Organoid domes were transferred into 96-well Lumitrac microplate (Greiner, 655075) and treated in 100 µl cell culture media at 37 °C in a humidified incubator. CellTiter-Glo® 3D cell viability assay (Promega Corporation, G9681) was carried out according to the manufacturer's protocol.

FAEE-induced acute pancreatitis in mice

FAEE-induced AP was evoked in wild-type (WT) FVB/N and CFTR KO mice according to Huang et al. [22]. The animals received two intraperitoneal injections of ethanol (1.35 g/bwkg) and palmitoleic acid (POA; 150 mg/bwkg) at 1-h intervals. Control animals received two intraperitoneal injection of equivalent volume vehicle (25% DMSO, 75% sterile water). Treated groups received one intraperitoneal dose of aurintricarboxylic acid (ATA) (5 mg/bwkg) in vehicle (25% DMSO, 75% sterile water) 90 min before the first injection of ethanol/POA or vehicle [23]. The severity of pancreatitis was assessed via analysis of histology sections, and the measurement of serum amylase activity.

Statistical analysis

Statistical analysis was performed with Graphpad Prism software. All data are expressed as means ± SEM as well as individual data points. For parametric tests unpaired *T* test, for nonparametric tests Mann–Whitney test, Kruskal–Wallis test and Dunn's multiple comparison were used based on the normality of data distribution. *P* value below 0.05 was considered statistically significant.

Results

Absence of CFTR impairs the function of plasma membrane Ca^{2+} pump in pancreatic ductal epithelial cells

Our hypothesis was that the decreased CFTR expression caused by chronic ethanol per se is sufficient to disturb the Ca^{2+} homeostasis of the gastrointestinal epithelial cells. Previously, we established that acute exposure to ethanol releases Ca^{2+} from the ER and activates extracellular Ca^{2+} influx in pancreatic ductal cells [4]. To assess whether the decreased CFTR expression disturbs the intracellular Ca^{2+} homeostasis wild-type (WT) and *Cftr* KO mice pancreatic ducts were challenged with carbachol. The maximal Ca^{2+} release was not different between the two groups, the slope of the Ca^{2+} signal plateau phase—representing the Ca^{2+} extrusion from the cytosol—was significantly higher in *Cftr* KO ductal fragments compared to WT (Fig. 1A). Next, we utilized mouse pancreatic organoids (MPO) generated from WT and *Cftr* KO mice. WT organoids were treated with 100 mM ethanol (EtOH) and 200 µM palmitic acid (PA) for 12 h, control and *Cftr* KO MPOs received no treatment. Store-operated Ca^{2+} influx was activated by re-addition of the extracellular Ca^{2+} after ER depletion (25 µM cyclopiazonic-acid (CPA) in Ca^{2+}-free media) (Fig. 1B.i). The basal intracellular Ca^{2+} concentrations were significantly higher in the EtOH-treated and *Cftr* KO organoids (Fig. 1B.ii). As expected, the ER Ca^{2+} release in response to CPA was lower in the EtOH-treated organoids and was not changed in *Cftr* KO organoids (Fig. 1B.iii), whereas both EtOH-treated and *Cftr* KO organoids showed a significantly decreased Ca^{2+} extrusion after removal of the extracellular Ca^{2+} (Fig. 1B. iv). The same phenomenon was observed in *Cftr* KO ducts (Supplementary Fig. 1A). Next human pancreatic organoids (HPO) were treated with 100 mM EtOH and 200 µM PA overnight. Importantly, compared to untreated HPOs, Ca^{2+} extrusion was significantly decreased after pre-incubation with EtOH-PA (Fig. 1C). Next, to confirm that the observed difference in Ca^{2+} extrusion was specific to CFTR-expressing cells, we analyzed Ca^{2+} signaling in pancreatic acinar cells, which lack CFTR in general [24] and did not detect difference in the carbachol response (maximal intracellular Ca^{2+} release or extrusion) between WT and *Cftr* KO mice acini (Supplementary Fig. 1B). Moreover, functional inhibition of CFTR with 10 µM CFTR(inh)-172—which significantly impaired CFTR activity (Supplementary Fig. 1C) [4]—had no effect on the carbachol-induced Ca^{2+} extrusion in WT ductal cells (Fig. 1D). Correction of CFTR expression in CFPAC-1 cells [14]—derived from liver metastasis of a CF patient's pancreatic ductal adenocarcinoma—restored Ca^{2+} extrusion (Supplementary Fig. 2A, B.). In contrast,

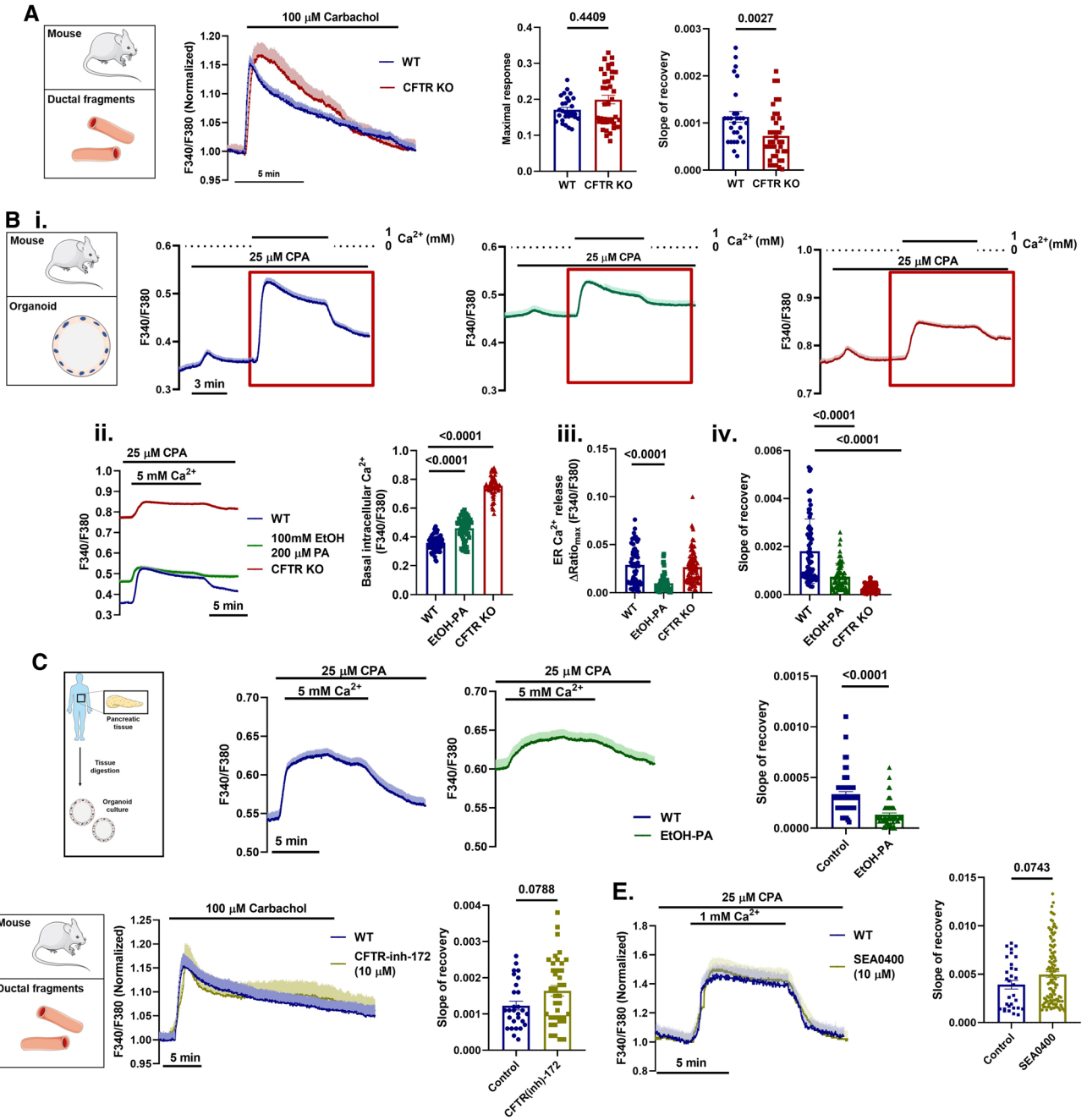


Fig. 1 Lack of CFTR expression leads to impaired Ca^{2+} extrusion in mouse pancreatic ductal cells. **A** Average traces of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), maximal Ca^{2+} elevation, and the slope of recovery in wild-type (WT) and *Cftr* KO pancreatic ductal fragments in response to 100 μM carbachol. **B** Average $[Ca^{2+}]_i$ traces show the Ca^{2+} efflux after ER Ca^{2+} store depletion (red quadrant highlights the Ca^{2+} extrusion used to calculate the slope of recovery) of mouse pancreatic ductal organoids (i). The basal intracellular Ca^{2+} levels were elevated (ii), whereas the Ca^{2+} extrusions (slope of recovery) were significantly reduced (iv) both in ethanol-palmitic acid (EtOH-PA) pre-treated and *Cftr* KO organoids. **C** Average traces and bar chart show that the Ca^{2+} extrusion was significantly decreased in EtOH-PA pre-treated human pancreatic organoids. **D** Average traces of $[Ca^{2+}]_i$ and the slope of recovery demonstrate that inhibition of CFTR function has no effect on the intracellular Ca^{2+} signaling. **E** Average traces and bar chart demonstrating that Na^+/Ca^{2+} exchanger (NCX) inhibition with SEA0400 had no effect on the Ca^{2+} extrusion in pancreatic ductal cells $n = 4–10$

knockdown of CFTR expression in WT ductal fragments with siCFTR impaired Ca^{2+} extrusion compared to control (Supplementary Fig. 2C–D.). Considering that both PMCA and Na^+/Ca^{2+} exchangers (NCX) can forward Ca^{2+} extrusion in non-excitable cells, we used the pan-NCX inhibitors SEA0400 and CB-DMB to assess the contribution of NCX

to the process. None of these inhibitors had any effect on the slope of the decrease (Fig. 1E; Supplementary Fig. 2E). Recently, partner of STIM1 (POST)—an adaptor protein linking STIM1 to other proteins—was shown to enhance the function of PMCA4 [25]. However, siSTIM1 treatment had no effect on the Ca^{2+} efflux in WT pancreatic ducts, suggesting that Stim1-POST is not involved in the regulation of PMCA in epithelial cells (Supplementary Fig. 2F). Taken together, these results indicate that attenuation of CFTR expression -rather than the lack of activity- by ethanol treatment is sufficient to alter Ca^{2+} homeostasis through limiting PMCA activity.

Ethanol has no effect on the PMCA4 expression in pancreatic ductal cells.

Currently, four mammalian PMCA genes have been identified which contribute to cytosolic Ca^{2+} extrusion [26]. Using whole transcriptome analysis, we revealed the expression of *Pmca1* and *Pmca4* in MPO and *PMCA1* and *PMCA4* in HPO samples, with highest levels of *Pmca1* in mouse and highest levels of *PMCA4* in humans (Fig. 2A,

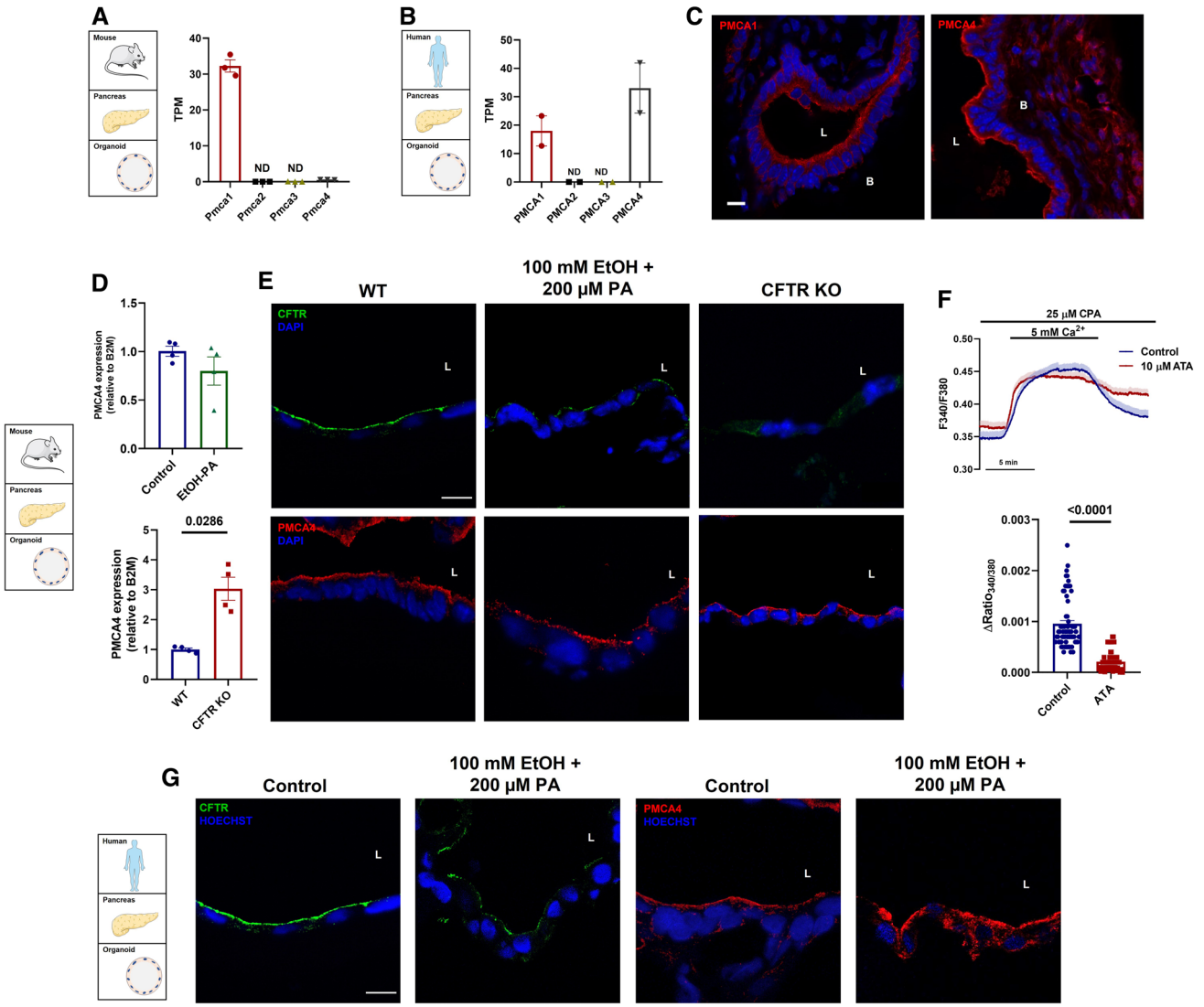


Fig. 2 Ethanol has no effect on the PMCA4 expression of pancreatic ductal cells. Whole transcriptome analysis of **A** mouse (MPO) and **B** human pancreatic organoids (HPO) demonstrate the expression of *Pmca1* and *Pmca4* (given in TPM: transcripts per million) $n=3$. **C** Localisation of PMCA1 and PMCA4 in mouse pancreatic organoids. **D** PMCA4 gene expression in MPOs relative to Beta-2-microglobulin (B2M). Pre-incubation with EtOH-PA had no effect whereas *Cftr* knockdown significantly increased the *Pmca4* expression. **E** Confocal images of MPOs show even, apical localisation of CFTR and PMCA4. This apical CFTR distribution was significantly impaired in *Cftr* KO and EtOH-PA pre-treated organoids. PMCA4 localization was not changed in MPO. Scale bar = 10 µm. **F** PMCA4 function of MPOs was significantly reduced by 30 min pre-incubation with 10 µM aurintricarboxylic acid (ATA) in vitro. **G** CFTR and PMCA4 are localized on the apical membrane of HPOs. This apical CFTR distribution was significantly impaired in *Cftr* KO and EtOH-PA pre-treated organoids, whereas increased cytosolic staining of PMCA4 was observed

B). Of note, expression levels of *Pmca2* and *Pmca3* were below detection limit in all samples. RT-PCR followed by endpoint analysis confirmed the expression of *Pmca1* and *Pmca4* in whole pancreatic tissue as well as isolated mouse pancreatic ducts (Supplementary Fig. 3A). Immunofluorescent staining of PMCA1 and PMCA4 in cross-sections of isolated mouse pancreatic ducts revealed the apical localization of PMCA4, whereas PMCA1 was evenly distributed over the apical and basolateral membranes (Fig. 2C). In addition, a strong co-localization of PMCA4 and CFTR at the apical membrane was observed (Mander's correlation coefficient: 0.906, Supplementary Figs. 3B) in pancreatic ductal epithelial cells. In intestinal stem cells, loss of CFTR expression results in alkaline pH_i deriving Wnt/β-catenin-mediated expression of different genes [27], which may affect the expression of PMCA4 in pancreatic ductal cells. To test this, the relative expression of *Pmca4* was compared with qRT-PCR in control, EtOH-PA-treated and *Cftr* KO MPOs. While, control and EtOH-PA-treated WT MPO showed no significant alteration, *Pmca4* expression was moderately increased in *Cftr* KO ductal organoids compared to WT control suggesting that the difference of Ca^{2+} efflux is not due to reduced gene expression (Fig. 2D). Similar moderate changes were observed in the Pmca1 expression in EtOH-PA-treated and *Cftr* KO MPOs (Supplementary Fig. 3C). Next, we wondered whether loss of CFTR due to EtOH treatment would alter the apical membrane-specific localisation of PMCA4. Whereas immunofluorescent microscopy revealed diminished CFTR levels at the apical membrane in EtOH-PA-treated and *Cftr* KO MPOs compared to untreated WT, PMCA4 retained its apical localisation in all samples (Fig. 2E). Next, to confirm the interaction of CFTR and PMCA4 on the functional level, we pre-incubated MPOs in vitro for 30 min with 10 µM aurintricarboxylic acid (ATA), a selective PMCA4 inhibitor, which significantly impaired the intracellular Ca^{2+} extrusion (Fig. 2F). However, no difference was observed between pre-treated WT and CFTR KO organoids, suggesting that PMCA1 function is not affected by the lack of CFTR (Supplementary Fig. 3D), therefore, in the downstream analysis, we focused on PMCA4. Subsequently, the presence of CFTR and PMCA4 on the apical plasma membrane of HPOs was confirmed by immunolabelling. Whereas overnight incubation of HPO with EtOH-PA resulted in a diminished, patchy apical expression pattern of CFTR, PMCA4 retained its apical membrane localisation (Fig. 2G). Notably, alcohol treatment resulted in a detectable cytosolic shift of PMCA4. These results suggest that the lack of CFTR at the apical membrane of pancreatic ductal cells diminishes the activity but not the expression or localization of PMCA4.

iPSC-derived organoids from cystic fibrosis patients recapitulate the alteration of PMCA function

Our results suggest that the diminished CFTR expression caused by genetic mutations in CF may also disturb Ca^{2+} extrusion of pancreatic ductal cells. Therefore, we assessed the relevance of our findings in human iPSC-derived pancreatic organoids generated from CF patients [20]. To establish CF-iPSC lines from donors affected by classical CF, lentiviral reprogramming of patient keratinocytes was used as previously described and performed stepwise in vitro differentiation to direct the iPSCs towards the pancreatic lineage followed by generation of exocrine pancreatic organoids in 3D-suspension culture (Fig. 3A). First, immunofluorescent analysis revealed that, while CFTR levels were absent in CF patient-derived organoids, which was markedly restored by 12 h incubation with the CFTR-corrector VX-809 (10 µM), PMCA1 and PMCA4 expression was present in control- and CF patient-derived iPSC organoids (Fig. 3B, Supplementary Fig. 4). Then, Ca^{2+} removal after ER Ca^{2+} store depletion resulted in a significantly decreased Ca^{2+} extrusion in CF organoids compared to control, further recapitulating our previous observation obtained in other model systems (Fig. 3C). Importantly, pre-treatment with 10 µM VX-809 for 12 h significantly improved Ca^{2+} extrusion indicating that CFTR-corrector treatment can restore decreased PMCA activity and thus the Ca^{2+} extrusion in CF organoids.

Ethanol reduces CFTR expression and PMCA activity in cholangiocytes

Although the cholangiocyte secretory function greatly depends on CFTR activity [8], alcohol-related changes in CFTR function or expression were never analyzed in alcoholic hepatitis (AH). Immunohistochemistry on formalin-fixed paraffin-embedded liver samples revealed that the apical CFTR distribution in cholangiocytes was significantly impaired in patients with AH compared to controls (Fig. 4A). Next, we recapitulated this phenomenon in vitro in WT mouse-derived liver organoids (MLO) positive for the epithelial cell lineage marker KRT19 (Supplementary Fig. 5A). CFTR showed a luminal membrane localisation in untreated MLOs, which was significantly decreased and shifted towards the cytosol in EtOH-treated MLOs without biologically relevant changes in *Cftr* gene expression levels (Fig. 4B; Supplementary Fig. 5B). Subsequent functional analysis of MLOs revealed a significantly impaired apical Cl^-/HCO_3^- exchange activity in EtOH-treated MLOs compared to control (Fig. 4C). In addition, whereas extracellular Cl^- removal resulted in CFTR-dependent increase in MQAE fluorescence—used as a marker of intracellular Cl^- [17]—in control MLOs, alcohol treatment resulted in

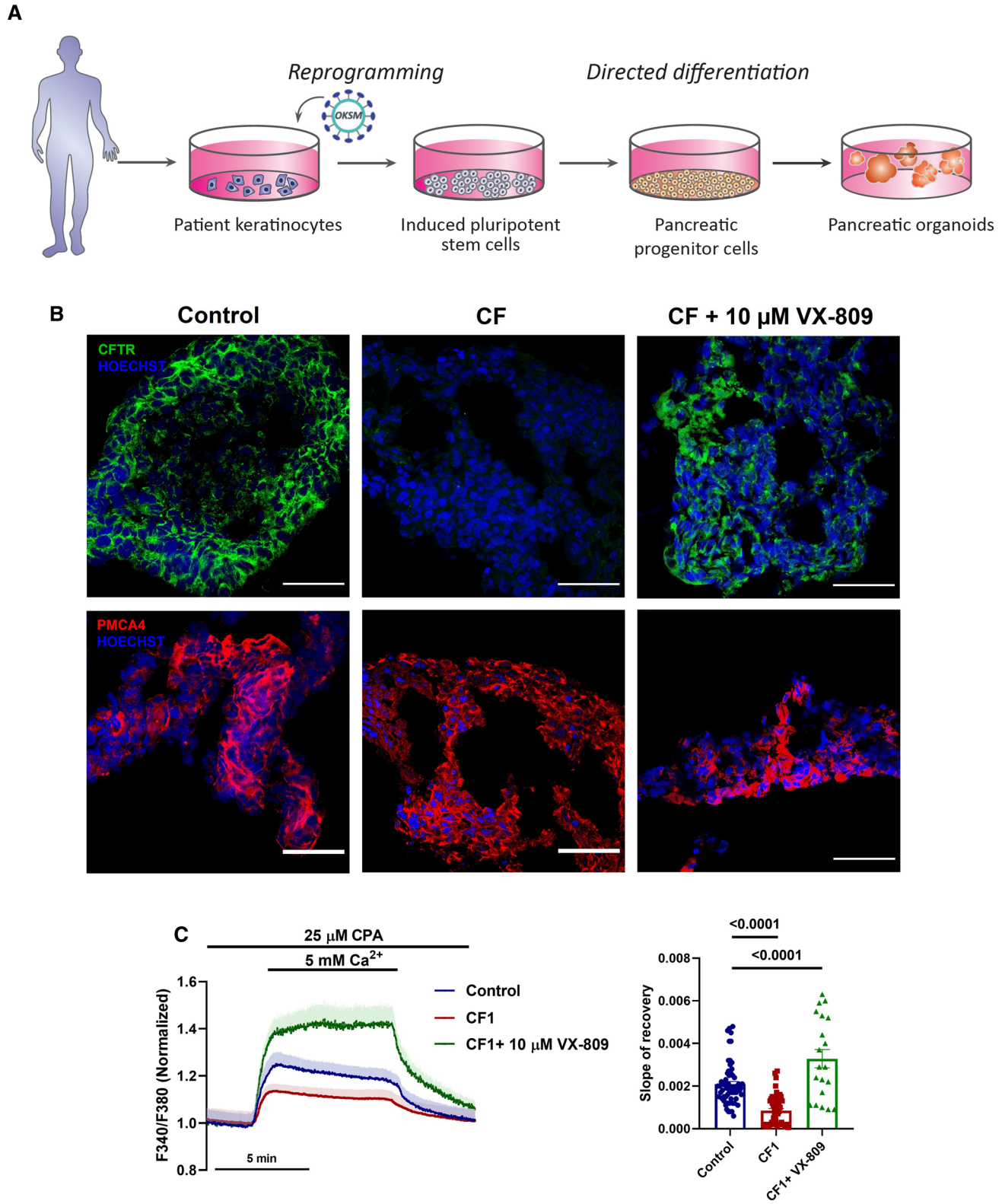


Fig. 3 PMCA4 activity is decreased in cystic fibrosis human pancreatic organoids. **A** Schematic outline of the generation of induced pluripotent stem cell (iPSC)-derived pancreatic organoids from cystic fibrosis (CF). **B** Confocal pictures confirm the presence of both CFTR and PMCA4 in control iPSC organoids, while CF organoids showed no staining for CFTR. 12 h incubation with 10 µM VX-809 restored CFTR expression in CF organoids. **C** Average traces and bar charts highlight a significant decrease in the Ca^{2+} extrusion in CF organoids compared to control, which was markedly improved after VX-809 treatment. Scale bars = 20 µm. $n = 4$–10 individual experiments

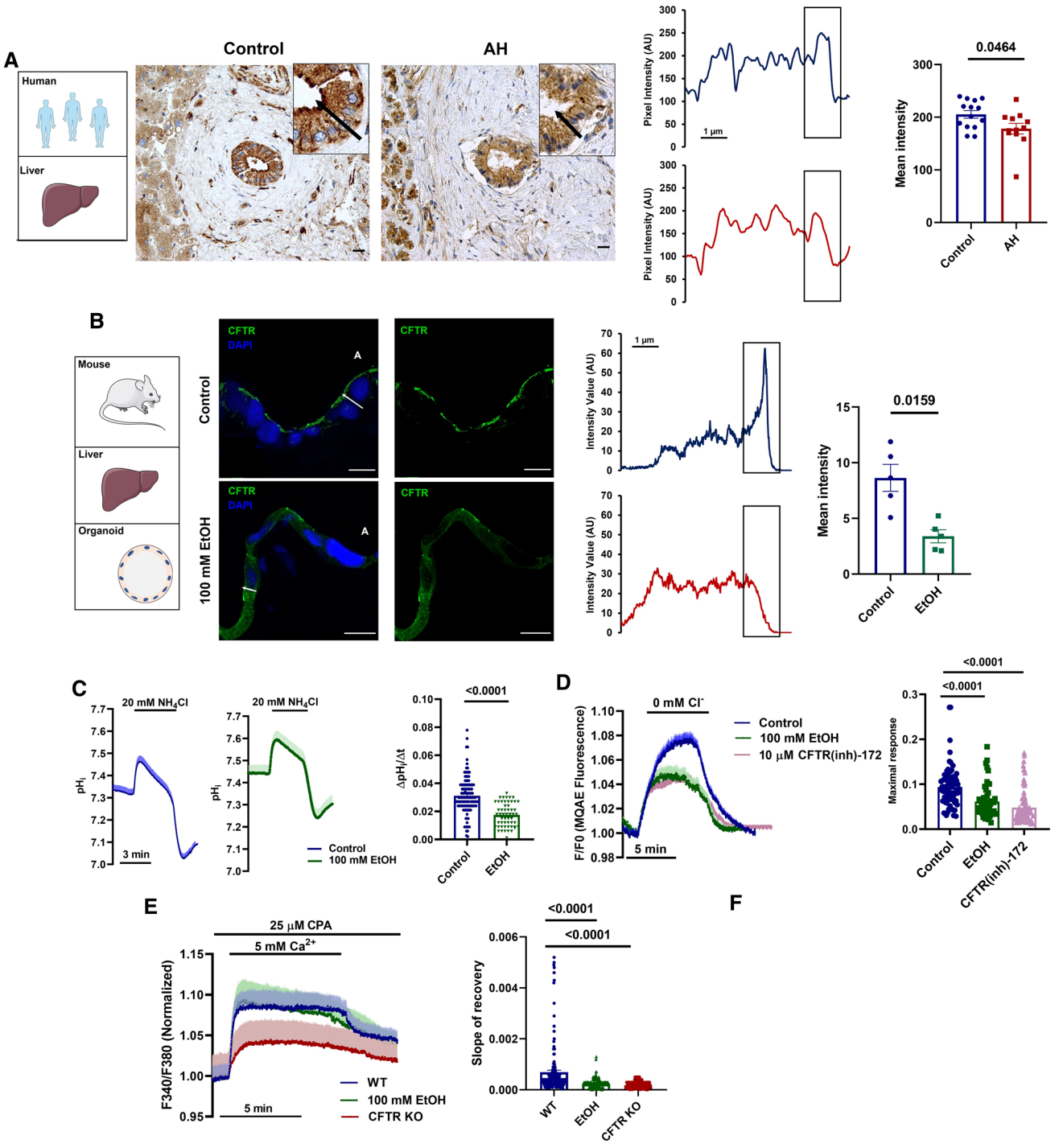


Fig. 4 CFTR expression and PMCA4 activity is impaired in cholangiocytes in alcoholic hepatitis. **A-B** CFTR expression of human cholangiocytes (**A**) and mouse liver organoids (**B**). In control cholangiocytes and organoids, CFTR is localized on the apical plasma membrane (black boxes). In patients with alcoholic hepatitis (AH) and ethanol-palmitic acid (EtOH-PA) pre-treated organoids, the apical distribution significantly decreased. Scale bar = 10 µm. L: lumen. **C** Average traces show the regeneration from alkalosis in liver organoids after exposure to 20 mM NH_4Cl in HCO_3^-/CO_2-buffered solution. The base flux from alkali load represents the activity of the apical anion exchange, which was significantly decreased in EtOH-PA-treated liver organoids. **D** Average MQAE traces show the CFTR-mediated Cl^- efflux after Cl^- withdrawal from the extracellular solution. The activity of CFTR was significantly reduced by overnight incubation of the liver organoids with EtOH-PA. **E** Average traces and bar charts demonstrate that the Ca^{2+} extrusion is decreased in EtOH-PA pre-treated and in *Cftr* KO mouse liver organoids. **F** *PMCA4* gene expression was not changed in EtOH-PA pre-treated and in *Cftr* KO mouse liver organoids. $n = 4–6$ individual experiments

a significant decrease of CFTR-dependent Cl^- extrusion (Fig. 4D). Finally, Ca^{2+} measurements revealed significantly decreased PMCA activity in ethanol pre-incubated—as well as *Cftr* KO organoids compared to WT control, suggesting that decreased apical distribution of CFTR impairs PMCA function in cholangiocytes (Fig. 4E). Changes of *Pmca4* gene expression did not achieve a biologically relevant level in MLOs (Fig. 4F). Importantly, these results highlight that EtOH exposure alters CFTR localization and activity in cholangiocytes leading to decreased ion secretion and disturbed intracellular Ca^{2+} homeostasis.

PMCA4 interacts with CFTR at the apical membrane of pancreatic ductal epithelial cells

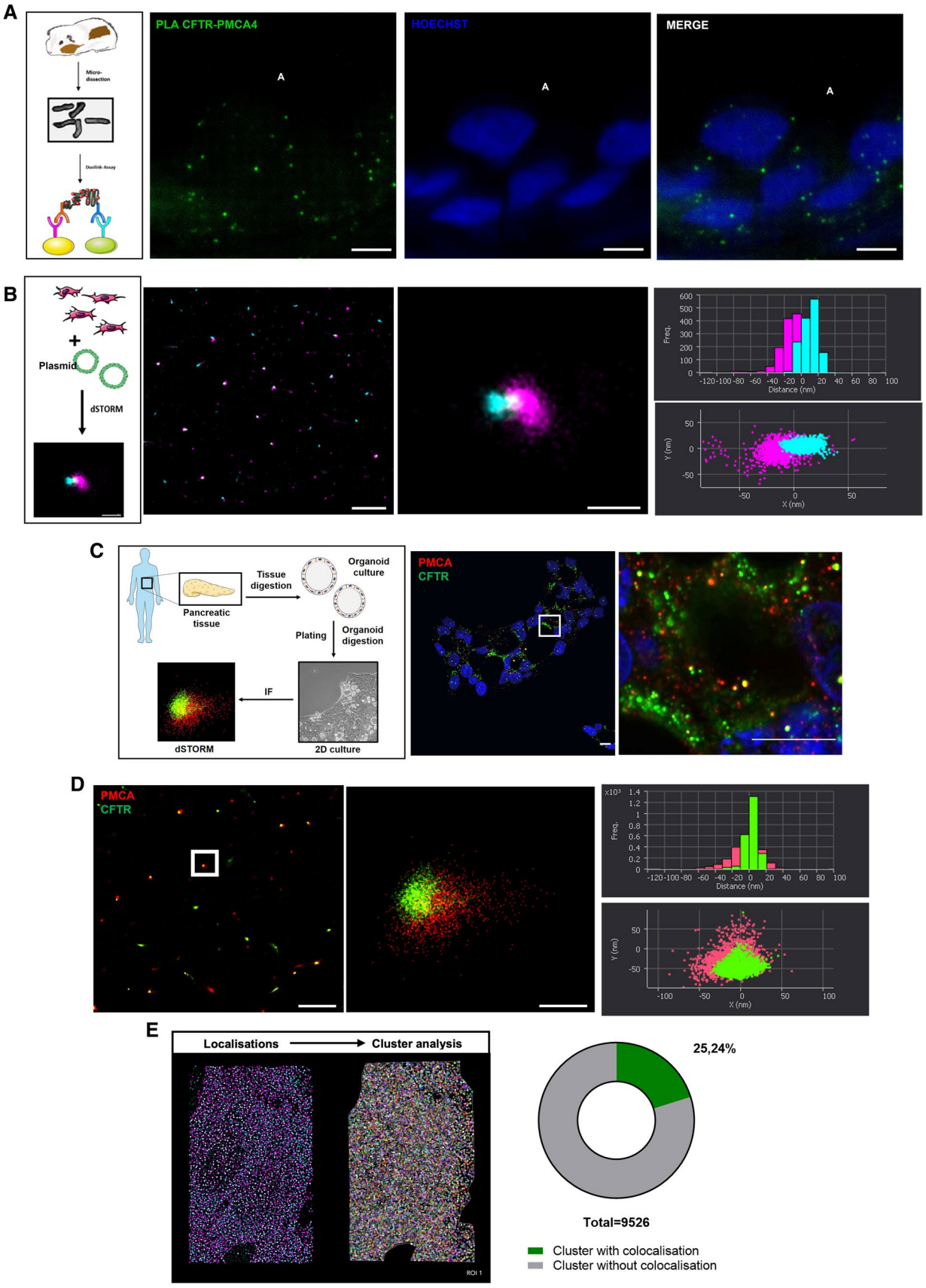
Our observations suggesting that proper PMCA4 activity requires a close connection with CFTR. Therefore, we performed Duolink proximity ligation assay (PLA) between endogenous PMCA and CFTR. Of note, to avoid non-specific antibody binding, guinea pig pancreatic ductal fragments were used, which recapitulated the co-localization of PMCA4 and CFTR (Supplementary Fig. 6A). Duolink PLA suggested that PMCA4 and CFTR are in a proximity of < 40 nm (Fig. 5A). Then, we used dSTORM to visualize this interaction at even higher resolution. First, in HeLa cells co-transfected with plasmids encoding CFTR and PMCA4, we observed a perfect overlap (< 20 nm) between the two proteins in the plasma membrane suggesting physical proximity (Fig. 5B, Supplementary Fig. 6B). Next, we established 2D adherent primary human ductal cell culture from pancreatic ductal organoids, which was suitable for dSTORM imaging. In these cells, we confirmed overlapping localisation patterns of endogenously expressed CFTR and PMCA4 with confocal microscope (Fig. 5C) and dSTORM (Fig. 5D, Supplementary Fig. 6C). Of note, cluster analysis of the dSTORM images revealed a co-localization of 25.24% between all clusters of endogenously expressed CFTR and PMCA4 in 2D human pancreatic ductal cells (Fig. 5E).

Calmodulin binding by CFTR regulates PMCA4 activity in pancreatic ductal cells and in cholangiocytes

Next, we wanted to provide mechanistic insight into the regulation of PMCA4 activity by CFTR. The recently described alternative calmodulin binding of CFTR has been suggested to allow the regulation of other proteins [12]. Thus, we hypothesized that such type of calmodulin-CFTR interaction might subsequently influence the activity of the calmodulin-regulated PMCA4. First, we evidenced strong co-localization of calmodulin with CFTR and PMCA4 at the apical membrane of ductal epithelial cells with dSTORM on cross-sections of MPOs (Supplementary Fig. 7). Next, whereas calmodulin strongly associated with the apical membrane in WT MPOs and MLOs, it dissociated from the apical membrane and diffused throughout the cytosol—as suggested by the line intensity profiles—in EtOH-treated or *Cftr* KO MPOs and MLOs (Fig. 6A, B). A similar localisation pattern was observed in *Cftr* KO ductal fragments (Supplementary Fig. 8). Then, we wanted to analyze the effect of impaired calmodulin-CFTR interaction on PMCA4 activity in epithelial cells. As general knockdown or inhibition of calmodulin can have multiple downstream effects, we co-transfected HEK-293 cells with PMCA4 and CFTR or CFTR harboring a mutation in the calmodulin-binding site (CFTR(S768A)). Of note, both CFTR and CFTR(S768A) localized to the plasma membrane and co-localized with PMCA4 (Supplementary Fig. 9A, B.). While co-transfection of PMCA4 and CFTR markedly increased the slope of Ca^{2+} extrusion, PMCA4 alone showed moderate activity (Fig. 6C). However, more importantly, cells transfected with CFTR(S768A) showed a significantly impaired PMCA4 activity compared to cells transfected with CFTR. Moreover, dSTORM cluster analysis revealed a 34% reduction of the co-localization ratio between PMCA4-CFTR(S768A) compared to PMCA4-CFTR (Fig. 6D, E, Supplementary Fig. 9C) suggesting that the lack of calmodulin/CFTR interaction is sufficient to decrease PMCA4 activity as well as the stability of the protein nanodomain on the apical plasma membrane.

Inhibition of PMCA4 impairs mitochondrial function, increases apoptosis, and results in more severe ethanol-induced acute pancreatitis

Sustained intracellular Ca^{2+} elevation is known to impair mitochondrial function and trigger apoptosis [28]. In the next step, we wanted to assess the role of impaired CFTR expression in this phenomenon. Transmission electron microscopy showed no difference in the mitochondrial volume/cell ratio between *Cftr* KO and WT pancreatic ductal cells (Supplementary Fig. 10A). Next, administration of 100 µM carbachol resulted in a significant decrease in mitochondrial membrane potential ($\Delta\psi_m$) in EtOH-PA pre-treated and *Cftr* KO—but not in WT—MPOs and MLOs, suggesting that a sustained intracellular Ca^{2+} elevation impairs mitochondrial function in pancreatic ductal cells and cholangiocytes (Fig. 7A, B). This was further tested by comparing the total ketone body concentration in WT and CFTR KO mice (Supplementary Fig. 10B). According to our results, the total ketone body concentration was increased in the CFTR KO mice, however this difference was not significant. To further establish the connection between the impaired CFTR expression and mitochondrial function, we measured the intracellular ATP levels in WT control, EtOH-PA-treated


A
Micro-dissection
Duolink-Assay
PLA CFTR-PMCA4
HOECHST
MERGE
B
Plasmid
dSTORM
Freq.
Distance (nm)
Y (nm)
X (nm)
C
Pancreatic tissue
Tissue digestion
Organoid culture
Plating
Organoid digestion
IF
dSTORM
2D culture
PMCA
CFTR
D
PMCA
CFTR
Freq.
Distance (nm)
Y (nm)
X (nm)
E
Localisations
Cluster analysis
ROI 1
25,24%
Total=9526
Cluster with colocalisation
Cluster without colocalisation

◂**Fig. 5** Interaction of PMCA4 with CFTR at the apical plasma membrane. **A** Endogenous PMCA and CFTR are in close proximity (< 40 nm) as suggested by Duolink proximity ligation assay. (Merged image of 18 optical sections). Scale bar = 10 µm. **B** dSTORM images of HeLa cells transfected with CFTR and PMCA4 revealed a perfect overlap (< 20 nm) between the two proteins in the plasma membrane. Scale bars = 1 µm and 50 nm, respectively. **C** Confocal images of 2D adherent primary ductal cells generated from human pancreatic ductal organoids. Scale bars = 5 µm. **D** dSTORM images of primary human pancreatic ductal cells revealed 25.24% cluster co-localization of endogenous PMCA4 and CFTR clusters. Scale bars = 1 µm and 50 nm, respectively. 5–7 cells were analyzed for each condition

and CFTR KO MPOs (Fig. 7C). This test also confirmed that both EtOH-PA treatment and the lack of CFTR expression impaired the mitochondrial ATP production and thus the cell viability. To function properly, the ATPase PMCA4 relies on ATP generated by oxidative phosphorylation and glycolysis. As EtOH decreases the mitochondrial ATP production [4], we inhibited the F1F0-ATPase by oligomycin, which had no effects on the PMCA function in ductal cells (Supplementary Fig. 10C). However, the intracellular distribution of cytochrome c released from the mitochondria—a hallmark of apoptosis—significantly increased in *Cftr* KO compared to WT pancreatic ductal cells, suggesting that sustained Ca^{2+} elevation and disturbed mitochondrial function leads to apoptosis (Supplementary Fig. 10D). In addition, *Cftr* KO pancreatic ductal cells had higher cytoplasmic levels of the initiator caspase 9 compared to WT pancreatic ductal cells, further confirming the increased rate of apoptosis (Supplementary Fig. 10E). Finally, using the PMCA4 inhibitor aurintricarboxylic acid (ATA) in an alcohol-induced pancreatitis mouse model, we aimed to analyze if impaired PMCA4 function could independently enhance the severity of pancreatic and liver diseases. The effect of pre-incubation of pancreatic ductal organoids with 10 µM ATA has been shown above. Interestingly, ATA treatment significantly impaired *Pmca4* expression as well, whereas *Pmca1* expression was not changed (Supplementary Fig. 11A, B). Next, a single injection of ATA (intraperitoneally, 5 mg/kg) was administered to WT FVB/N mice 90 min before the first EtOH-POA injection [23]. Compared to vehicle control, ATA pre-treated animals had significantly elevated pancreatic edema and necrosis scores paralleled with significantly elevated serum amylase activities (Fig. 7D). Importantly, CFTR KO mice displayed more severe experimental AP in response to EtOH-PA injection compared to the WT animals, which was not increased further by the ATA treatment (Supplementary Fig. 11C–F.). Taken together, these results indicate that impaired PMCA4 activity diminish mitochondrial function, augments apoptosis, and potentially increases the severity of CFTR-related pancreatic- and presumably liver diseases.

Discussion

In this study, we provided evidence that impaired CFTR expression induced by ethanol exposure or genetic mutations subsequently decreased CFTR-mediated recruitment of calmodulin to the apical membrane attenuates PMCA4 activity and Ca^{2+} extrusion in pancreatic ductal cells and cholangiocytes. The consequent disturbed Ca^{2+} homeostasis leads to damaged mitochondrial function and enhanced apoptosis and ultimately results in increased disease severity. Thus, our results shed light on a novel regulatory mechanism of intracellular Ca^{2+} signaling that might contribute to severity of alcoholic pancreatitis- and hepatitis and potentially to the development of CF-related liver and pancreatic damage.

CFTR is generally considered as a cAMP-activated Cl^- channel, although it may be regulated by $[Ca^{2+}]_i$, or by possible interactions with other components involved in Ca^{2+} homeostasis. However the downstream effects of these interactions on the subcellular signaling events or on the activity of CFTR is not well understood [29, 30]. In our study, we first demonstrated that Ca^{2+} extrusion is significantly impaired in ethanol-treated and *Cftr* KO pancreatic ductal cells due to diminished PMCA activity. Using several independent in vitro model systems, including HPOs and iPSC-derived organoids generated from CF patients, we revealed that the lack of apical CFTR—rather than its function—affects PMCA activity. The fact that diminished CFTR expression did not influence PMCA4 expression or cellular distribution implied a functional interaction between the two proteins. Previously, altered intracellular Ca^{2+} signaling due to elevated IP_3R-dependent Ca^{2+} release and SERCA activity as well as decreased PMCA function was described in cultured bronchial epithelial cells that express F508del CFTR [9, 10]. In addition, CF cells display enhanced mitochondrial Ca^{2+} uptake compared to controls [31]. Importantly, correction of CFTR expression with VX-809 in this model seemed to restore the intracellular Ca^{2+} signaling alterations [32]. Besides increased intracellular Ca^{2+} release and impaired Ca^{2+} clearance, increased activity of extracellular Ca^{2+} influx is described in CF. Antigny et al. [33] demonstrated that transient receptor potential canonical 6 (TRPC6) channel-dependent extracellular Ca^{2+} influx is increased in CF airway cells. Moreover, Balghi et al. [34] indicated that Orai1-mediated extracellular Ca^{2+} influx is equally increased in CF airway cells leading to increased secretion of the proinflammatory cytokine IL-8. On the other hand, sustained intracellular Ca^{2+} overload is a hallmark of AP. The most frequent pathogenic factors—including bile acids or non-oxidative EtOH metabolites—trigger the release of ER Ca^{2+} stores and activate extracellular

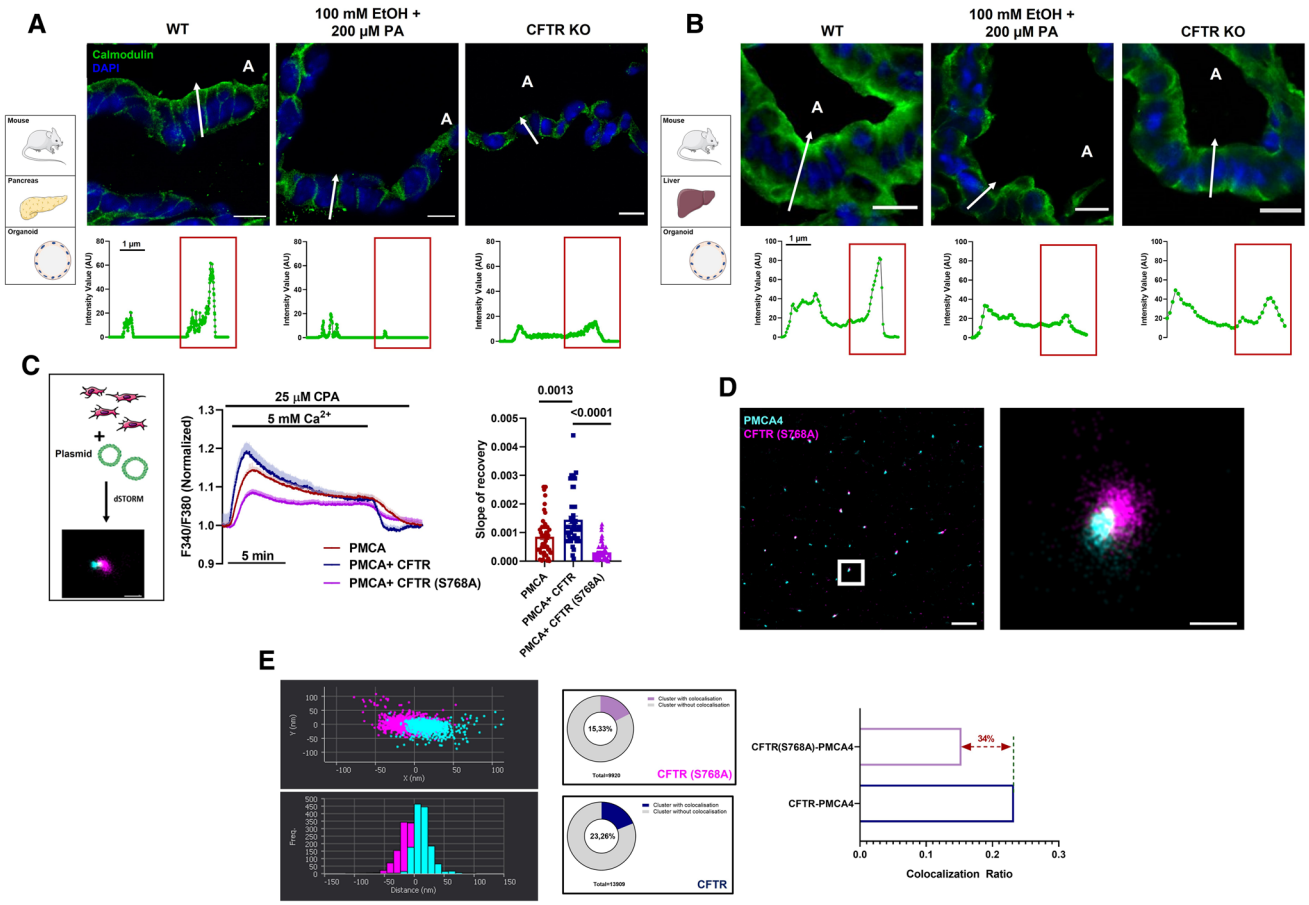


Fig. 6 Apical calmodulin recruitment by CFTR regulates PMCA4 activity. **A-B** Confocal images and line intensity profiles demonstrate the apical membrane association of calmodulin in wild-type (WT) pancreatic and liver organoids. In ethanol/palmitic acid (EtOH-PA)-treated WT and *Cftr* KO cells, this association was lost. Scale bar = 10 µm. **C** Co-transfection of PMCA4 and CFTR markedly increased the slope of Ca^{2+} extrusion, whereas the calmodulin-binding site mutant CFTR(S768A) significantly impaired the activity of PMCA4. $n = 4–6$ individual experiments. **D** Cluster analysis of dSTORM images revealed 34% reduction of the co-localization ratio in CFTR(S768A)-PMCA4 compared to CFTR-PMCA4. Scale bars = 1 µm and 50 nm, respectively. 5–7 cells were analyzed for each condition

Ca^{2+} influx leading to impaired fluid and HCO_3^- secretion [35, 36]. The subsequent sustained intracellular Ca^{2+} overload triggers mitochondrial damage with consequent ATP depletion and cell damage [21], further impairing ATP-dependent Ca^{2+} extrusion.

AH is a potentially lethal complication of alcoholic liver disease, which has been attributed to hepatocellular damage in the past [5]. Recently, binding of neutrophils to ITGB1 expressed on the cell surface of cholangiocytes was shown to contribute to epithelial cell damage and the development of cholestasis in AH [7]. Other studies showed that cholestatic liver injury can be involved in the pathogenesis of AH, moreover, impaired secretion by cholangiocytes, or cholestasis, results in a worse outcome [6]. In addition, other liver diseases—such as primary biliary cholangitis, primary sclerosing cholangitis, or cystic fibrosis-related liver disease (CFRD)—are associated with dysfunctional cholangiocyte secretion [37]. This previously unrecognized contribution of cholangiocyte damage in AH altered our understanding of the disease pathogenesis and offers novel therapeutic strategies. A previous study described that stimulation of Toll-like receptor 4 by lipopolysaccharides activates NF-κB to down-regulate type 3 inositol trisphosphate receptor expression in human cholangiocytes, which may contribute to the cholestasis observed in AH [37]. However, this was not directly caused by ethanol exposure. Proper cholangiocyte secretion largely depends on efficient functioning of CFTR located at the apical membrane of these cells [8, 38]. As EtOH and its metabolites damage the expression and function of CFTR in pancreatic ductal epithelial cells [4], it seems plausible that alcohol-induced CFTR damage may contribute to the development of cholangiocyte dysfunction and cholestasis in AH. In this study, we demonstrated significantly decreased apical plasma membrane CFTR expression

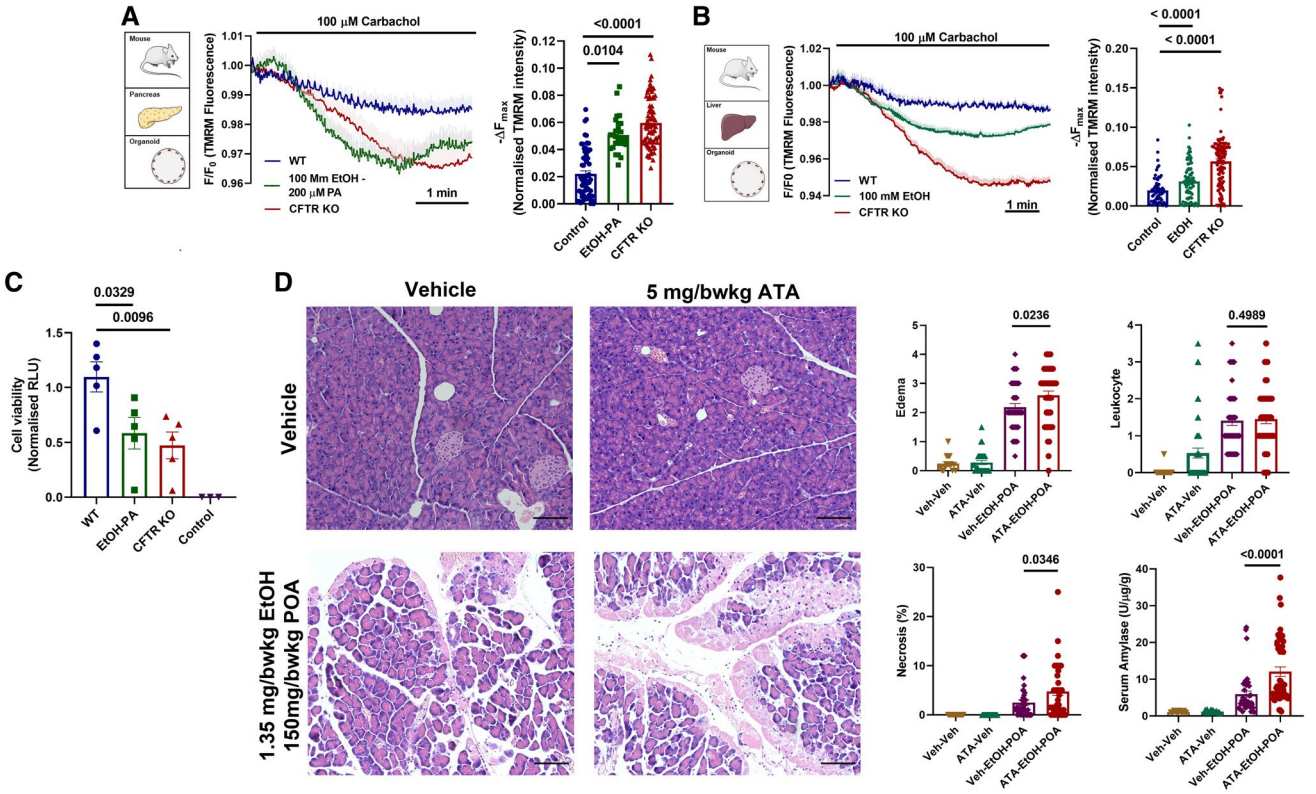


Fig. 7 Decreased PMCA4 activity impairs mitochondrial function, increases apoptosis, and the severity of alcohol-induced acute pancreatitis in mice. **A-B** Average traces of mitochondrial membrane potential ($\Delta\psi_m$) and maximal fluorescent intensity changes in response to 100 µM carbachol show a significant decrease in the $\Delta\psi_m$ in ethanol-palmitic acid (EtOH-PA) pre-treated WT and *Cftr* KO mouse pancreatic and liver organoids compared to WT. $n = 6–10$ individual experiments. **C** CellTiter-Glo 3D cell viability assay revealed significant decrease of ATP production in EtOH-PA pre-treated WT and CFTR KO MPOs compared to WT. **D** Representative hematoxylin and eosin (H&E) images, histology scores, and serum amylase activities demonstrate the severity of alcohol-induced AP in mice. In vivo treatment with 5 mg/bwkg ATA significantly elevated the serum amylase activities, oedema, and necrosis scores. $n = 6–18$ animals/group

accompanied with a significantly impaired CFTR activity in post-mortem AH patient liver samples and apical diminished Cl^-/HCO_3^- exchange upon alcohol exposure in mouse liver organoids. In addition, both EtOH pre-incubated WT and *Cftr* KO liver organoids displayed impaired Ca^{2+} extrusion due to decreased PMCA activity. The lack of CFTR-mediated secretion as well as altered intracellular Ca^{2+} homeostasis can damage the cholangiocyte secretion contributing to the development of AH-related cholestasis and potentially CF-related liver disease (CFLD) and severely worsen the clinical outcome.

In our experiments, we demonstrated that PMCA4 co-localizes with CFTR at the apical membrane of ductal cells in multiple models. Moreover, using super-resolution dSTORM, we highlighted that CFTR and PMCA4 are within 20 nm distance suggesting a physical interaction of the two proteins. Previously, the co-immunoprecipitation experiments of Philippe et al. [10] suggested that CFTR interacts with SERCA and PMCA in airway epithelial cells, although the nature of this interaction was not revealed. Recently, Bozoky et al. [12] demonstrated increased open probability of CFTR due to direct binding of calmodulin to its R domain, which provided a novel mechanism for the regulation of intracellular Ca^{2+}-mediated CFTR activity. Interestingly, the authors proved that calmodulin binds to CFTR in an alternative binding conformation allowing the two lobes of calmodulin to independently bind two separate sequences. This binding conformation may allow CFTR to recruit calmodulin and subsequently determine the activity of other calmodulin-regulated proteins such as PMCA isoforms in a macromolecular complex at the apical plasma membrane. Binding of Ca^{2+}-calmodulin with the calmodulin-binding domain of PMCA competitively antagonizes the autoinhibitory domain leading to PMCA activation [39]. In untreated WT pancreatic- and liver organoids, calmodulin was associated with the apical membrane and strongly co-localized with CFTR and PMCA4. In *Cftr* KO- and ethanol-treated organoids, this apical localization was lost suggesting that the presence of CFTR at the apical membrane is necessary to recruit calmodulin. To verify that interaction of calmodulin with CFTR is required for PMCA activation, we overexpressed a calmodulin-binding site mutant of CFTR

[CFTR(S768A)] and PMCA4 in HEK-293 cells and showed significantly impaired Ca^{2+} extrusion, whereas the interaction between CFTR and PMCA4 also remarkably decreased.

During the pathogenesis of alcohol-induced AP, it is well established that acute exposure of acinar [40] and ductal cells [4] to ethanol or ethanol metabolites induce sustained elevation of $[Ca^{2+}]_i$ leading to the opening of the mitochondrial permeability transition pore dissipating $\Delta\Psi_m$ with a consequent drop of ATP synthesis [41, 42]. In our experiments, instead of acute administration, we incubated the ductal cells overnight, which may model the effects ethanol consumption better. Although we found no evidence for mitochondrial morphological damage but detected a remarkable drop of $\Delta\Psi_m$ when challenging the ethanol-treated and *Cftr* KO MPOs and MLOs with carbachol, which was accompanied by the decrease of mitochondrial ATP production as well. In addition, increased cytosolic staining for cytochrome c and caspase 9 in *Cftr* KO ductal cells suggested increased apoptosis. Finally, PMCA4 inhibition in an in vivo model of alcoholic AP significantly increased disease severity suggesting that impaired PMCA4 function due to impaired CFTR expression by itself can contribute to cell damage in alcoholic pancreatitis and hepatitis. Importantly, our results highlight that restoration of PMCA activity or enhancement of the Ca^{2+} extrusion can have potential therapeutic benefit not only in alcoholic pancreatitis and hepatitis, but also in cystic fibrosis-related liver disease and pancreatitis. We demonstrated that restoration of CFTR expression with correctors can also improve the function of PMCA. Effectivity of triple combination therapy for CF patients was shown recently [43] and persistent improvement of pancreatic function was reported in CF patients receiving ivacaftor [44]. Moreover, prevention of intracellular Ca^{2+} overload with pharmacologic inhibition of Orai1 resulted in favorable clinical outcomes among patients with severe AP [45]. As an example, in a recent study the phenothiazine methylene blue (MB) was shown to stimulate PMCA activity in neural cultures and in human tissues from Alzheimer's disease-affected brain [46]. As MB is used to treat methemoglobinemia and other diseases [47], it could be utilized in alcohol-induced hepatitis and pancreatitis and in CFLD and pancreatic diseases as well.

Taken together, our results revealed that decreased CFTR expression impairs PMCA4 function and results in sustained intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation in ethanol-treated mouse and in human pancreatic ductal organoids. Moreover, AH patient liver samples and ethanol-treated liver organoids showed decreased CFTR expression, and impaired PMCA4 activity. CFTR co-localizes with PMCA4 on the apical membrane of polarized epithelial cells and regulates PMCA4 activity in a calmodulin-dependent manner. Finally, sustained intracellular Ca^{2+} elevation due to impaired PMCA4 activity in the absence of CFTR inhibits mitochondrial function, increases apoptosis and the severity of alcohol-induced AP in mice. Thus, our results suggest that prevention of sustained intracellular Ca^{2+} overload by improving Ca^{2+} extrusion may protect the exocrine pancreatic function in alcoholic pancreatitis and prevent the development of cholestasis in AH resulting in an improved disease outcome.

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Author contributions TM and JM: designed the research project; TM, ÁV, NP, VSZ, BD, BT, PP, JF, RZ, ZR, MH, AK, and TL: contributed to acquisition, analysis and interpretation of data for the work; TM and JM: drafted the work, PH, MG and ZR: revised the manuscript critically for important intellectual content. All the authors approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Data availability The datasets generated during and/or analyzed during the current study are fully available upon contact with the corresponding author.

Declarations

Conflict of interest None of the authors has conflict of interest to declare.

Ethics approval Animals were used with adherence to the NIH guidelines and the EU directive 2010/63/EU. The study was approved by the National Scientific Ethical Committee on Animal Experimentation under license number XXI./2523/2018. The collection and use of human samples including cadaver donor pancreas and liver samples were executed in adherence with the EU standards and approved by

the Regional Committee of Research Ethics of the Hungarian Medical Research Council under license number 37/2017-SZTE.

References

1. World Health Organization. (2018) Global status report on alcohol and health 2018.
2. Yadav D, Lowenfels AB (2013) The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology 144:1252–1261
3. Párniczky A et al (2016) Prospective, multicentre, nationwide clinical data from 600 cases of acute pancreatitis. PLoS ONE 11:0165309
4. Maléth J et al (2015) Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. Gastroenterology 148:27-439.e16
5. Lucey MR et al (2009) Alcoholic hepatitis. N.Engl J. Med. 360:2758–2769
6. Altamirano J et al (2014) A histologic scoring system for prognosis of patients with alcoholic hepatitis. Gastroenterology 146:1231-1239.e1–6
7. Takeuchi M et al (2021) Neutrophils interact with cholangiocytes to cause cholestatic changes in alcoholic hepatitis. Gut 70:342–356
8. Trampert DC, Nathanson MH (2018) Regulation of bile secretion by calcium signaling in health and disease. Biochim Biophys Acta Mol Cell Res 1865:1761–1770
9. Antigny F et al (2008) Abnormal spatial diffusion of Ca2+ in F508del-CFTR airway epithelial cells. Respir Res 9:70
10. Philippe R et al (2015) SERCA and PMCA pumps contribute to the deregulation of Ca2+ homeostasis in human CF epithelial cells. Biochim Biophys Acta 1853:892–903
11. Maléth J, Hegyi P (2016) Ca2+ toxicity and mitochondrial damage in acute pancreatitis: translational overview. Philos Trans R Soc Lond B 371:20150425
12. Bozoky Z et al (2017) Synergy of cAMP and calcium signaling pathways in CFTR regulation. Proc Natl Acad Sci 114:E2086–E2095
13. Maléth J, Choi S, a.l. (2014) Translocation between PI(4,5) P2-poor and PI(4,5)P2-rich microdomains during store depletion determines STIM1 conformation and Orai1 gating. Nat Commun 5:5843
14. Rakonczay Z et al (2008) CFTR gene transfer to human cystic fibrosis pancreatic duct cells using a Sendai virus vector. J Cell Physiol 214:442–455
15. Ratcliff R et al (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. Nat Genet 4:35–41
16. Fanczal J et al (2020) TRPM2-mediated extracellular Ca2+ entry promotes acinar cell necrosis in biliary acute pancreatitis. J Physiol 598:1253–1270
17. Molnár R et al (2020) Mouse pancreatic ductal organoid culture as a relevant model to study exocrine pancreatic ion secretion. Lab. Investig. 100:84–97
18. Huch M et al (2013) In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 494:247–250
19. Breunig M et al (2021) Modeling plasticity and dysplasia of pancreatic ductal organoids derived from human pluripotent stem cells. Cell Stem Cell 28:1105-1124.e19
20. Hohwieler M et al (2017) Human pluripotent stem cell-derived acinar/ductal organoids generate human pancreas upon orthotopic transplantation and allow disease modelling. Gut 66:473–486
21. Maléth J et al (2011) Non-conjugated chenodeoxycholate induces severe mitochondrial damage and inhibits bicarbonate transport in pancreatic duct cells. Gut 60:136–138
22. Huang W et al (2014) Fatty acid ethyl ester synthase inhibition ameliorates ethanol-induced Ca2+-dependent mitochondrial dysfunction and acute pancreatitis. Gut 63:1313–1324
23. Lewis S et al (2018) Acute inhibition of PMCA4, but not global ablation, reduces blood pressure and arterial contractility via a nNOS-dependent mechanism. J Cell Mol Med 22:861–872
24. Yokoyama T et al (2019) Different immunohistochemical localization for TMEM16A and CFTR in acinar and ductal cells of rat major salivary glands and exocrine pancreas. Acta Histochem 121:50–55
25. Go CK et al (2019) Ca2+ export pump PMCA clears near-membrane Ca2+ to facilitate store-operated Ca2+ entry and NFAT activation. Sci. Signal. 12:eaaw2627
26. Stafford N et al (2017) The plasma membrane calcium ATPases and their role as major new players in human disease. Physiol Rev 97:1089–1125
27. Strubberg AM et al (2018) Cftr modulates Wnt/β-catenin signaling and stem cell proliferation in murine intestine. Cell Mol Gastroenterol Hepatol 5:253–271
28. Mukherjee R et al (2008) Mitochondrial injury in pancreatitis. Cell Calcium 44:14–23
29. Billet A, Hanrahan JW (2013) The secret life of CFTR as a calcium-activated chloride channel. J Physiol 591:5273–5278
30. Park S et al (2013) Irbit mediates synergy between ca(2+) and cAMP signaling pathways during epithelial transport in mice. Gastroenterology 145:232–241
31. Antigny F et al (2009) Dysfunction of mitochondria Ca2+ uptake in cystic fibrosis airway epithelial cells. Mitochondrion 9:232–241
32. Antigny F et al (2008) Calcium homeostasis is abnormal in cystic fibrosis airway epithelial cells but is normalized after rescue of F508del-CFTR. Cell Calcium 43:175–183
33. Antigny F et al (2011) Transient receptor potential canonical channel 6 links Ca2+ mishandling to cystic fibrosis transmembrane conductance regulator channel dysfunction in cystic fibrosis. Am J Respir Cell Mol Biol 44:83–90
34. Balghi H et al (2011) Enhanced Ca2+ entry due to Orai1 plasma membrane insertion increases IL-8 secretion by cystic fibrosis airways. FASEB J 25:4274–4291
35. Maléth J, Hegyi P (2014) Calcium signaling in pancreatic ductal epithelial cells: an old friend and a nasty enemy. Cell Calcium 55:337–345
36. Pallagi P, Madacsy T et al (2020) Intracellular Ca2+ signalling in the pathogenesis of acute pancreatitis: recent advances and translational perspectives. Int J Mol Sci 21:4005
37. Franca A et al (2019) Effects of endotoxin on type 3 inositol 1,4,5-trisphosphate receptor in human cholangiocytes. Hepatol 69:817–830
38. Guerra MT, Nathanson MH (2015) Calcium signaling and secretion in cholangiocytes. Pancreatol 15:S44-48
39. Hegedűs L et al (2020) Molecular diversity of plasma membrane Ca2+ transporting ATPases: their function under normal and pathological conditions. Adv Exp Med Biol 1131:93–129
40. Criddle DN et al (2006) Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. Gastroenterology 130:781–793
41. Petersen OH et al (2009) Fatty acids, alcohol and fatty acid ethyl esters: toxic Ca2+ signal generation and pancreatitis. Cell Calcium 45:634–642
42. Tóth E et al (2019) Novel mitochondrial transition pore inhibitor N-methyl-4-isoleucine cyclosporin is a new therapeutic option in acute pancreatitis. J Physiol 597:5879–5898
43. Barry PJ et al (2021) Triple therapy for cystic fibrosis phe508del-gating and -residual function genotypes. N.Engl J. Med. 385:815–825

44. Munce D et al (2020) Persistent recovery of pancreatic function in patients with cystic fibrosis after ivacaftor. Pediatr Pulmonol 55:3381–3383
45. Bruen C et al (2021) Auxora for the treatment of patients with acute pancreatitis and accompanying systemic inflammatory response syndrome: clinical development of a calcium release-activated calcium channel inhibitor. Pancreas 50:537–543
46. Berrocal M, Corbacho I, Gutierrez-Merino C, Mata AM (2018) Methylene blue activates the PMCA activity and cross-interacts with amyloid β-peptide, blocking Aβ-mediated PMCA inhibition. Neuropharmacology 139:163–172
47. PushparajahMak RS, Liebelt EL (2021) Methylene blue: an antidote for methemoglobinemia and beyond. Pediatr Emerg Care 37:474–477