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Original Articles

Defective CFTR promotes intestinal proliferation via inhibition of the hedgehog pathway during cystic fibrosis

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ABSTRACT

Hyperproliferation occurs in a variety of tissues and organs during cystic fibrosis (CF). However, the associated molecular mechanisms remain elusive. We investigated the molecular link between cystic fibrosis transmembrane conductance regulator (CFTR) defects and hyperproliferation, and showed that the length of the entire gastrointestinal tract was longer and the intestinal crypts were deeper in CF mice compared to those in wild-type animals. PCNA expression increased in CF mouse intestines and CFTR-knockdown cells. Villin1, an intestinal differentiation marker, was downregulated in CF mice. Ihh and Gli1 were significantly downregulated, whereas TCF4 was activated in CF mouse intestines and CFTR-knockdown Caco2 cells. Importantly, β-catenin activators rescued Gli1 suppression, suggesting that hedgehog signaling might be mediated by the Wnt/β-catenin pathway in the absence of functional CFTR. Moreover, PCNA positivity in the crypts of CF mice was alleviated by LiCl, which activates Wnt/β-catenin signaling. Further, a strong positive correlation was observed between the expression of CFTR and Ihh in intestines. Our study revealed a previously unidentified role of CFTR in regulating hedgehog signaling through β-catenin, providing novel insights into the physiological function of CFTR and CF-related diseases.

1. Introduction

Cystic fibrosis (CF), an autosomal recessive genetic disease that mostly occurs in Caucasians [1], is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes a cAMP-regulated anion channel at the apical membrane of epithelial cells of various organs [2]. Although more than 2000 mutations have been identified in CF (http://www.genet.sickkids.on.ca/cftr/

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ΔF508, deletion of phenylalanine at 508; Ihh, Indian hedgehog; PTCH, Patched; SMO, smoothened; Shh, Sonic hedgehog; Dhh, Desert hedgehog; ELISA, Enzyme-linked Immunosorbent Assays; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; H&E, Hematoxylin and Eosin; PBS, phosphate-buffered saline; RT-qPCR, real-time quantitative PCR

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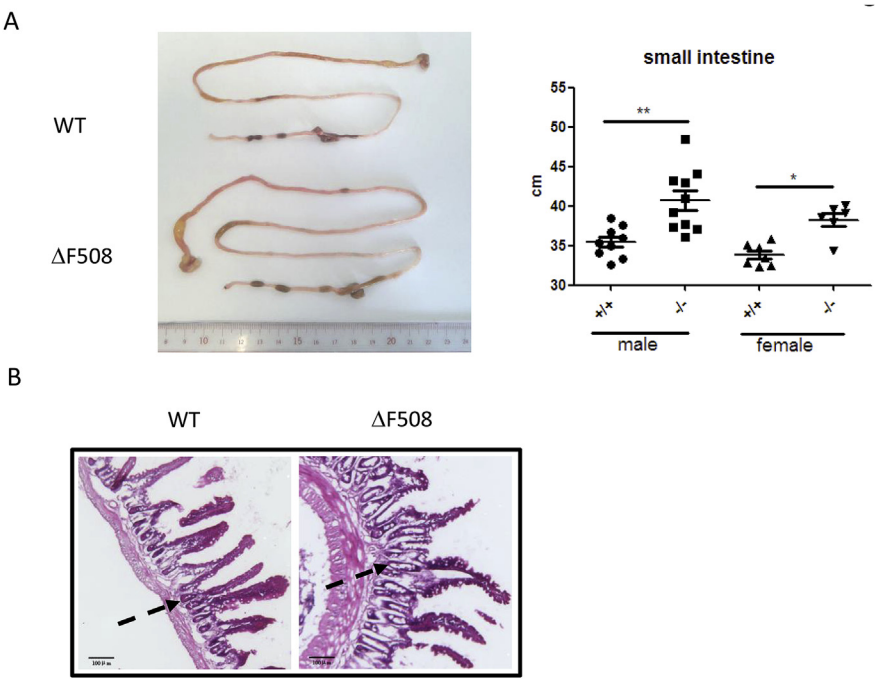

Fig. 1. The phenotype of the small intestine of ΔF508 mice.
(A) Representative picture showing the length of the small intestine in wild-type and CFTR ΔF508 mice (left). Quantification of mean intestine length (right; male: n = 18; female: n = 12). (B) H&E staining of small intestine section. The depth of the crypt (pointed with broken line) increased in the CFTR ΔF508 mouse small intestine. **p < 0.01, *p < 0.05.

app), deletion of phenylalanine at 508 (ΔF508), which results in a CFTR-folding defect and endoplasmic reticulum-associated degradation, is the most frequent mutation occurring in > 80% of CF patients [3,4]. *CFTR* mutations affect mucosal physiology of the respiratory, digestive, and reproductive tracts, leading to different clinical manifestations including pancreatic insufficiency, focal biliary cirrhosis, infertility, and chronic airway obstruction [5]. Particularly, there has been recent interest in the risk of various cancers in CF patients and *CFTR* mutation carriers [6–8]. For example, a study involving > 38,000 CF patients revealed increased incidence of digestive tract cancers including colorectal cancer [9]. Interestingly, *CFTR* is frequently hypermethylated in various cancer cell lines and clinical tumor samples [10,11], indicating that its loss might be critical for the pathogenesis of cancer. Moreover, epithelial cell proliferation was found to increase in the intestines of *CFTR*-mutant mice [12]. However, the exact mechanisms underlying this effect remains elusive.

Intestinal epithelium renewal is tightly controlled by Hedgehog genes. Three Hedgehog genes are highly conserved in mouse and human, including Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) [13]. Hedgehogs bind to Patched (PTCH) [14–16], which unlike conventional receptors, does not transmit the Hedgehog signal to the intracellular components but alleviates the inhibitory effect of PTCH on another membrane receptor, the seven-transmembrane protein Smoothened (SMO) [17–19]. In the absence of ligand (Ihh, Shh, or Dhh), Hh-ligand–receptor–PTCH suppresses SMO, and thus inhibits the cleavage of Gli [20,21]. When ligands bind PTCH, SMO suppression is relieved, and Gli is cleaved and transported to the nucleus resulting in the transcription of Hh target genes including PTCH and Gli themselves and cell proliferation-related genes including CyclinD1 [21,22]. PTCH1, PTCH2, Gli1, and Hedgehog-interacting protein can be used as readouts of pathway activity [23,24]. Ihh is produced by differentiated cells in the adult colon [25] and small intestine [26]. Evidence indicates that it acts as a negative feedback signal for proliferating cells in the crypt [27]. Thus, Hedgehog signaling might negatively regulate intestinal epithelium proliferation and reduce the transit amplifying region of the crypt in adult mice. However, the role of Ihh-mediated proliferation in CFTR-mutant intestines is unknown.

Wnt/β-catenin signaling is implicated in the control of stem cell activity, cell proliferation, and cell survival in the gastrointestinal epithelium. Interestingly, β-catenin has been shown to regulate the Ihh pathway in colon cancer [28,29]. We found that CFTR interacts with β-catenin and stabilizes it in epithelial cells and embryonic stem cells [30,31]. Given the reported involvement of Ihh in regulating intestinal proliferation, we hypothesized that CFTR might regulate Ihh activity through the β-catenin pathway, dysfunction of which might lead to inhibition of the hedgehog pathway and exaggerated proliferation, which is observed in *CFTR*-mutant intestines. We tested this hypothesis and focused on potential correlations between CFTR and the Ihh pathway.

2. Materials and methods

2.1. Animals

Cftr^{tm1Kth} (ΔF508) mice were purchased from Jackson's laboratory [32], which have a mutant *CFTR* gene with a 3-bp deletion that eliminates phenylalanine, which is the most frequent *CFTR* mutation in humans. They were maintained in an air-conditioned room with a controlled temperature of 24 ± 2 °C and humidity of 55 ± 15%, with a 12-h light/dark cycle, and were fed laboratory chow and water ad libitum. All animal experiments were conducted in accordance with the University Laboratory Animals Service Center's guidelines from the Animal Ethnics Committee of the University. Three-month-old *Cftr*^{tm1Kth} mice (wild-type and ΔF508) were used. Wild-type and ΔF508 mice were assigned into two groups (N = 4) treated with vehicle or LiCl. LiCl (200 mg/kg) was administrated by oral gavage for 8 days. At day 9, all mice were sacrificed by cervical dislocation. Intestines and colons were collected and stored in liquid nitrogen or fixed with 4% PFA for further use.

2.2. Antibodies and reagents

Anti-C-terminal-CFTR (CFTR-C) was from Alomone Labs (Jerusalem, Israel); anti-N-terminal-CFTR (CFTR-N) was from Millipore (Billerica, MA, USA). Anti-GAPDH, anti-Histone H1, anti-β-tubulin, anti-CyclinD1, and anti-PCNA were from Santa Cruz (Santa Cruz, CA, USA). Anti-Ihh, anti-SMO, anti-PTCH, anti-Lgr5, and anti-Gli1 were from Abcam (Cambridge, MA, USA). Anti-β-actin was from Sigma (St.

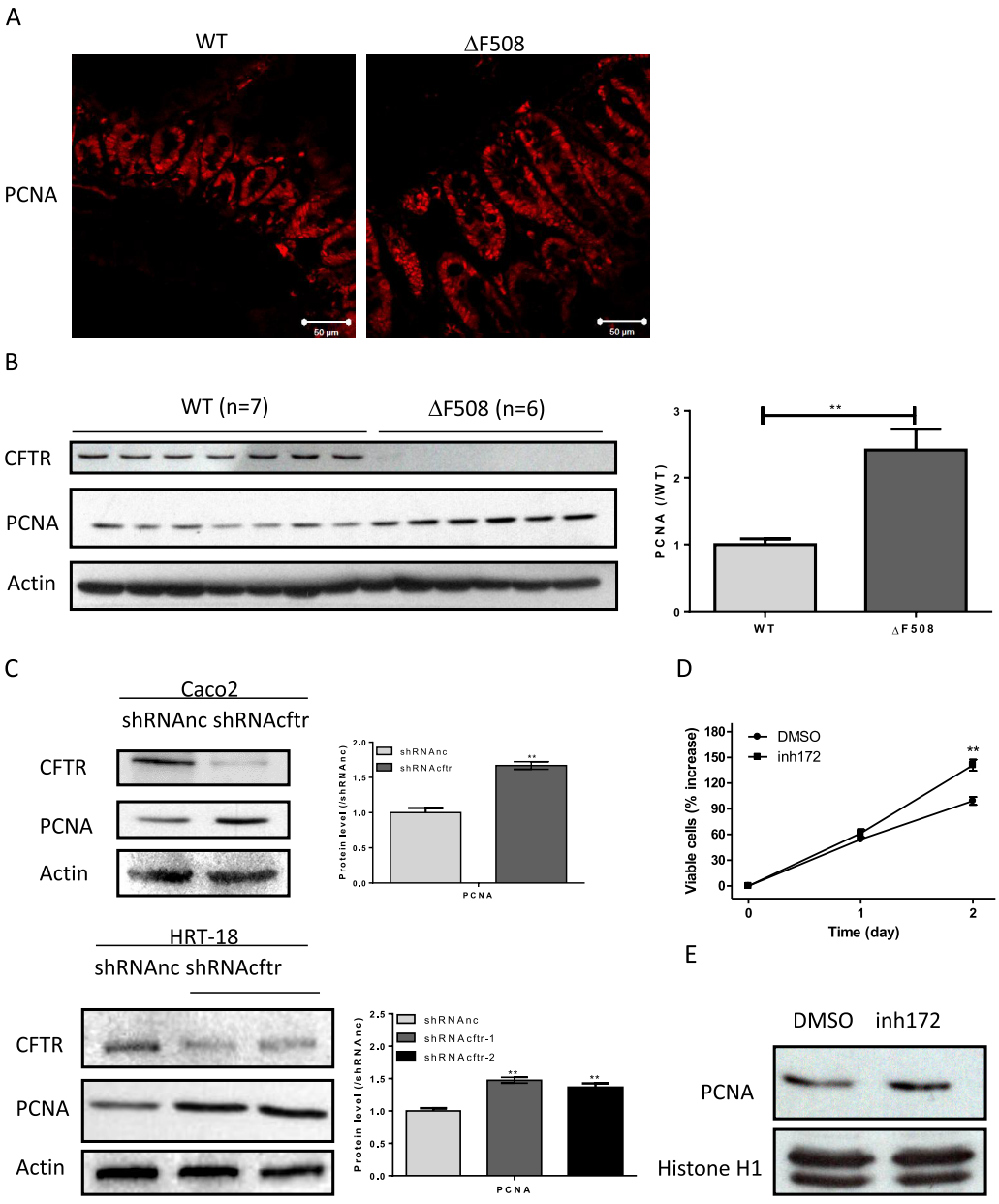

Fig. 2. Activation of proliferation in the ΔF508 mouse small intestine and after CFTR knockdown or inhibition in cells.
(A) Immunostaining for PCNA (red) in the small intestine of wild-type and CFTR ΔF508 mice. Scale bar = 50 μm. (B) Western blot analysis of CFTR and PCNA protein levels in the intestine. Actin was used as a loading control. The quantification of western blot results is shown in the right panel (n = 13). (C) Western blot analysis of PCNA in CFTR-knockdown Caco2 and HRT-18 cells. Actin was used as a loading control. The quantification of western blot results is shown in the right panel (n = 3). (D) MTT assay on IEC18 cells after CFTR inhibitor (10 μM inh172) treatment. The MTT results showed that the CFTR inhibitor promotes intestinal cell proliferation. (E) Western blot analysis of PCNA in CFTR-inhibited IEC18 cells. Histone H1 was used as a loading control. **p < 0.01.

Louis, MO, USA). Anti-villin1 and anti-TCF4 were from Cell Signaling Technology (Danvers, MA, USA). CHIR was obtained from Merck Millipore. Lithium chloride was obtained from Sigma (St. Louis, MO, USA).

2.3. Cell culture and lentiviral transduction

The rat intestinal cell line IEC18 and human colon cancer cell lines Caco2, HT29, HCT116, and HRT-18 (ATCC) were cultured in DMEM, MEM, or RPMI-1640, and supplemented with 10% FBS and 1% penicillin-streptomycin and maintained at 37 °C with 5% CO_2.

The β-catenin and Gli1 plasmids were purchased from Vigene biosciences (Jinan, China). siRNA oligos were synthesized by Jima Inc. (Shanghai, China). The sequences (5′–3′) of the three siRNA targeting TCF4 are GGGACAUGCAUGGAAUCAUTT, CUCAUCGUCUCCUAAUUA UTT, and GACGACAAGAAGGAUAUCATT. Lentiviral transduction particles encoding shRNA against *CFTR* were purchased from Jima Inc. (Shanghai, China). The sequences (5′–3′) of the two shRNA are GAAG TAGTGATGGAGAATGTA and TTGGAAAGGAGACTAACAAGT, respectively, which target different regions of human *CFTR* mRNA. Cells were plated in 24-well cell plates at 10^4 cells/well and incubated with 10 μl of lentivirus (1×10^9 TU/ml) for 8 h. Viral vectors containing non-coding shRNA served as controls. Transduced cells were expanded in 6-well cell culture plates, transferred to culture flasks, and selected using puromycin (10 μg/ml) for 2 weeks.

2.4. MTT assays

Cells (3×10^3/well) were plated in 96-well plates in 100 μl of media and cultured for 24, 48, or 72 h. After adding 20 μl of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution/well, the plates were incubated for 4 h, media were removed, formazan crystals were solubilized in 100 μl DMSO/well, and the absorbance was read at 570 nm.

2.5. H&E staining of cryosectioned samples

Sections were rinsed in tap water for 3 min, stained in hematoxylin solution for 10 min, rinsed in tap water for 1–2 min and differentiated in 1% acid ethanol for 2 s. Then, sections were washed in running tap

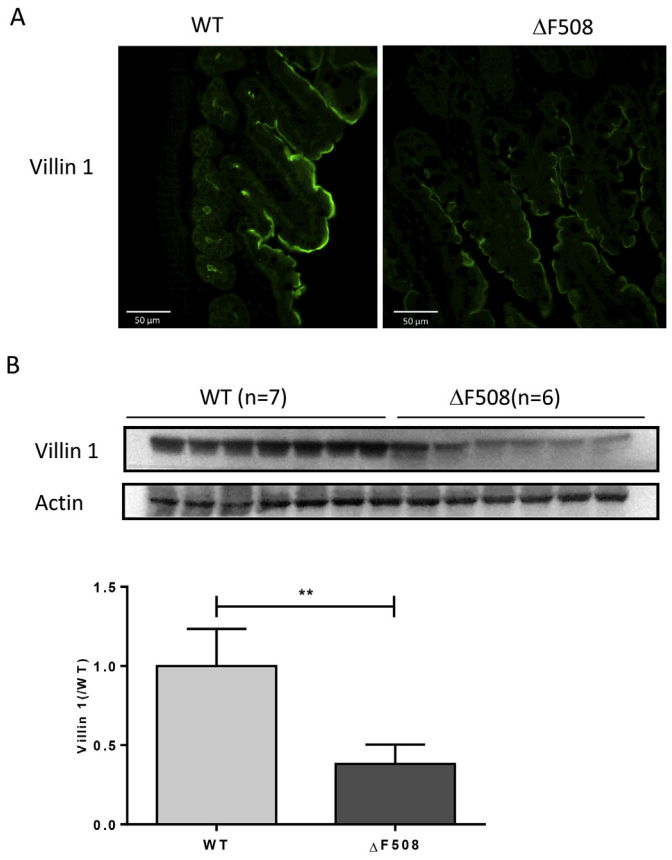

Fig. 3. Expression of Villin1 in the ΔF508 mouse small intestine.
(A) Immunostaining of Villin1 (Green) in the small intestine of wild-type and CFTR ΔF508 mice. Scale bar = 50 μm. (B) Western blot analysis of Villin1 protein levels in the intestine. Actin was used as a loading control. The quantification of western blot results is shown in the bottom panel (n = 13). **p < 0.01.

water for 2 min, blued in tap water for 10 min and stained in eosin Y solution for 1–2 min. After dehydrating with 95% ethanol (2 × 30 s), 100% ethanol (2 × 30 s), and cleared in xylene (2 × 10 min), sections were mounted using Permount (Fisher, USA).

2.6. Western blotting

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM tris–Cl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1:100 PMSF, and 1:100 protein inhibitors) for 30 min on ice. Supernatant was collected as total protein after centrifugation at 15,000 g for 30 min. Equal amounts of protein were separated by SDS-PAGE and subjected to western blotting. Protein bands were visualized using an enhanced chemiluminescence assay (GE Health) following the manufacturer's instructions and scanned using a densitometer. Experiments were repeated three times and bands were scanned and quantified.

2.7. Enzyme-linked immunosorbent assays (ELISA)

Caco2 cells were cultured overnight in 24 well plate, and then treated with or without 10 μM CFTR inh-172 or GlyH101 in MEM with 1% FBS for 24 h. Cells were washed with PBS three times, and cultured in 300 μl MEM only with or without inhibitors for 24 h. The medium was collected, and centrifuged with 300 g. The amounts of the hedgehog proteins in cell culture supernatants were determined by ELISA with an Ihh ELISA kit (Uscn Lifescience, Inc., Wuhan, China). All samples were examined in duplicate for each experiment.

2.8. Immunohistochemistry and fluorescent immunocytochemistry

Frozen mouse small intestine sections were prepared by cryostat sectioning. Sections were then incubated with PBS for 5–10 min and retrieval buffer (citrate buffer, pH 6.0) for 30 min. After washing with PBS, they were incubated with primary antibody overnight at 4 °C. The sections were then incubated with secondary antibody for 60 min at room temperature. Sections were mounted with coverslips and visualized by confocal laser scanning microscopy.

2.9. RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using RNeasy Mini Kit (QIAGEN GmbH, Germany) and was reverse transcribed using a PrimeScript RT Master Mix kit (TaKaRa, China) for standard real-time PCR analysis. RT-qPCR was performed using a TB Green Premix EX Taq II detection system in a Roche LightCycler 96 qPCR machine (Roche Diagnostics, Mannheim, Germany). β-actin was used as control. The gene-specific primers are summarized in Supplementary Table S1.

2.10. Primary colon cancer samples

To determine the clinical relevance of CFTR and Gli1 in colon cancer, tissue samples were collected from patients (n = 7) with primary colon cancer from the Department of gastrointestinal surgery, Shenzhen People's Hospital, Shenzhen, China. All samples were collected with patient informed consent, and the study was approved by the Ethics Committee.

A human intestine tissue microarray (SM2081, US Biomax Inc., USA) containing 95 cases were co-stained using mouse anti-CFTR (MAB3482, Millipore) and rabbit anti-Ihh (ab52919, Abcam). Immunofluorescence staining was based on the instructions of the manufacturer. In brief, antigen retrieval was performed on the deparaffinized array slide using 0.01 M sodium citrate buffer (pH 6.0) for 30 min at approximately 95 °C. Slides were washed twice with PBS and blocked with 2% normal donkey serum for 60 min at room temperature. Slides were incubated with CFTR (1:100) and Ihh (1:100) antibodies at 4 °C overnight, and then incubated with donkey-anti-mouse 488 and donkey-anti-rabbit 568 secondary fluorescent antibodies for 60 min at room temperature. The nucleus was stained with DAPI (4′, 6-diamidino-2-phenylindole, 5 μg/ml) for 15 min at room temperature. Slides were then mounted and inspected using a fluorescent microscope. Images of each sample were obtained using the same exposure parameters. Membrane and cytoplasmic CFTR signal intensity in intestinal epithelial cells was scored separately and then added as total CFTR expression. For Ihh expression, the epithelium, sub-epithelium, and goblet cells were scored and added. The score was from 0 to 10, indicating no signal and maximum signal intensity, respectively.

2.11. Statistical analysis

Data are expressed as mean ± SD. Differences in measured variables between two groups were analyzed by performing Student's t tests, and differences between more than two groups were analyzed by one-way ANOVA. Results were considered statistically significant at $P < 0.05$. (*$P < 0.05$, **$P < 0.01$).

3. Results

3.1. Small intestine length and crypt depth increases in ΔF508 mouse

In comparing phenotypic differences in the intestine between wild-type and ΔF508 mice, we showed that the length of the intestine in ΔF508 mice was significantly longer compared to that in wild-type mice for both males and females (Fig. 1A). The H&E staining also showed that crypt depth was increased (Fig. 1B).

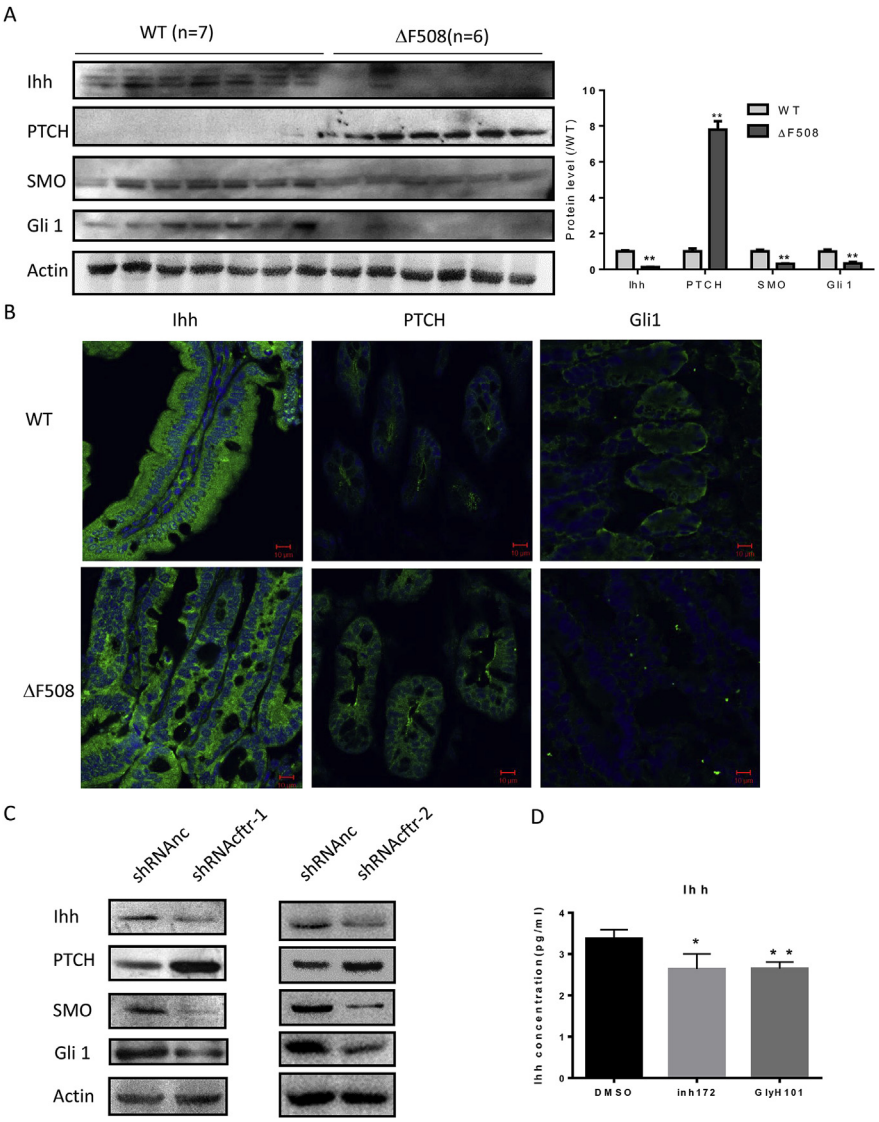

Fig. 4. Expression of the hedgehog pathway in the ΔF508 mouse small intestine and CFTR-knockdown Caco2 cells. (A) Western blot analysis of the hedgehog pathway in the small intestine of wild-type and CFTR ΔF508 mice. Results showed that Ihh, SMO, and Gli were downregulated, whereas PTCH was activated in the ΔF508 mouse small intestine. Actin was used as a loading control. The quantification of western blot results is shown in the right panel (n = 13). (B) Immunofluorescence staining of Ihh, PTCH, and Gli1 in the small intestine of wild-type and CFTR ΔF508 mice. Sections of intestine were stained with to-Pro3 (nuclear, blue), Ihh (Abcam, 1:100; green), PTCH (Abcam, 1:100; green), and Gli1 (Abcam, 1:100; green) antibodies. Scale bar = 10 μm. (C) Western blot analysis of the hedgehog pathway in CFTR-knockdown Caco2 cells. Actin was used as a loading control. (D) Secreted Ihh concentrations in CFTR inhibitor-treated Caco2 cells. Ihh secretion was inhibited after CFTR inhibition. $^{**}p < 0.01$, $^{*}p < 0.05$.

3.2. CFTR mutation leads to hyperproliferation in mouse small intestines and intestinal epithelial cell lines

To investigate if increased crypt depth was caused by increased proliferation, we stained for PCNA, a well-established proliferation marker. Results showed that PCNA-positive cells increased significantly in crypts of CFTR ΔF508 mice (Fig. 2A). Next, we analyzed PCNA protein levels in the intestine by western blotting. Results revealed that PCNA was upregulated significantly in the ΔF508 mouse small intestine indicating increased proliferation in the crypts (Fig. 2B). PCNA was also upregulated in *CFTR*-knockdown Caco2 and HRT-18 cells (Fig. 2C), indicating that knockdown of CFTR induces proliferation. We then analyzed proliferation and PCNA expression in IEC18 cells after CFTR inhibitor treatment. Results showed that this inhibitor could promote cell proliferation (Fig. 2D) and upregulate PCNA (Fig. 2E), suggesting that inhibition or downregulation of CFTR promotes proliferation.

3.3. ΔF508 mutation suppresses enterocyte maturation in the intestine

As an intestinal epithelial differentiation marker [33,34], we analyzed the localization and expression of Villin1 in ΔF508 mouse small intestines. Immunostaining showed Villin1 expression at the apical epithelial membrane of wild-type villi, which was remarkably reduced with the ΔF508 mutation (Fig. 3A). Western blotting also showed that Villin1 was downregulated significantly in ΔF508 mouse intestines (Fig. 3B).

3.4. ΔF508 suppresses hedgehog signaling in mouse small intestines and intestinal epithelial cells

To investigate the hedgehog pathway, we performed western blotting to analyze key proteins in this pathway. Results showed that Ihh, SMO, and Gli1 were downregulated, whereas PTCH was upregulated in the ΔF508 mouse small intestine (Fig. 4A). We further confirmed the differential expression of Ihh, PTCH and Gli1 in the small intestines of CF mice. Results showed that Ihh expression on the villi was downregulated. PTCH expression on the crypt cell membrane was upregulated and that Gli1 expression in the crypt cell cytoplasm was decreased significantly in CFTR ΔF508 mouse small intestines (Fig. 4B). Furthermore, knockdown of CFTR in Caco2 cells also decreased Ihh, SMO, and Gli1 protein levels and increased the protein level of PTCH (Fig. 4C). In addition, Ihh concentrations in the medium were detected by ELISA, and secreted Ihh was inhibited after blocking CFTR in Caco2 cells (Fig. 4D). Together, these results indicate that downregulation/mutation of CFTR inhibits the hedgehog pathway.

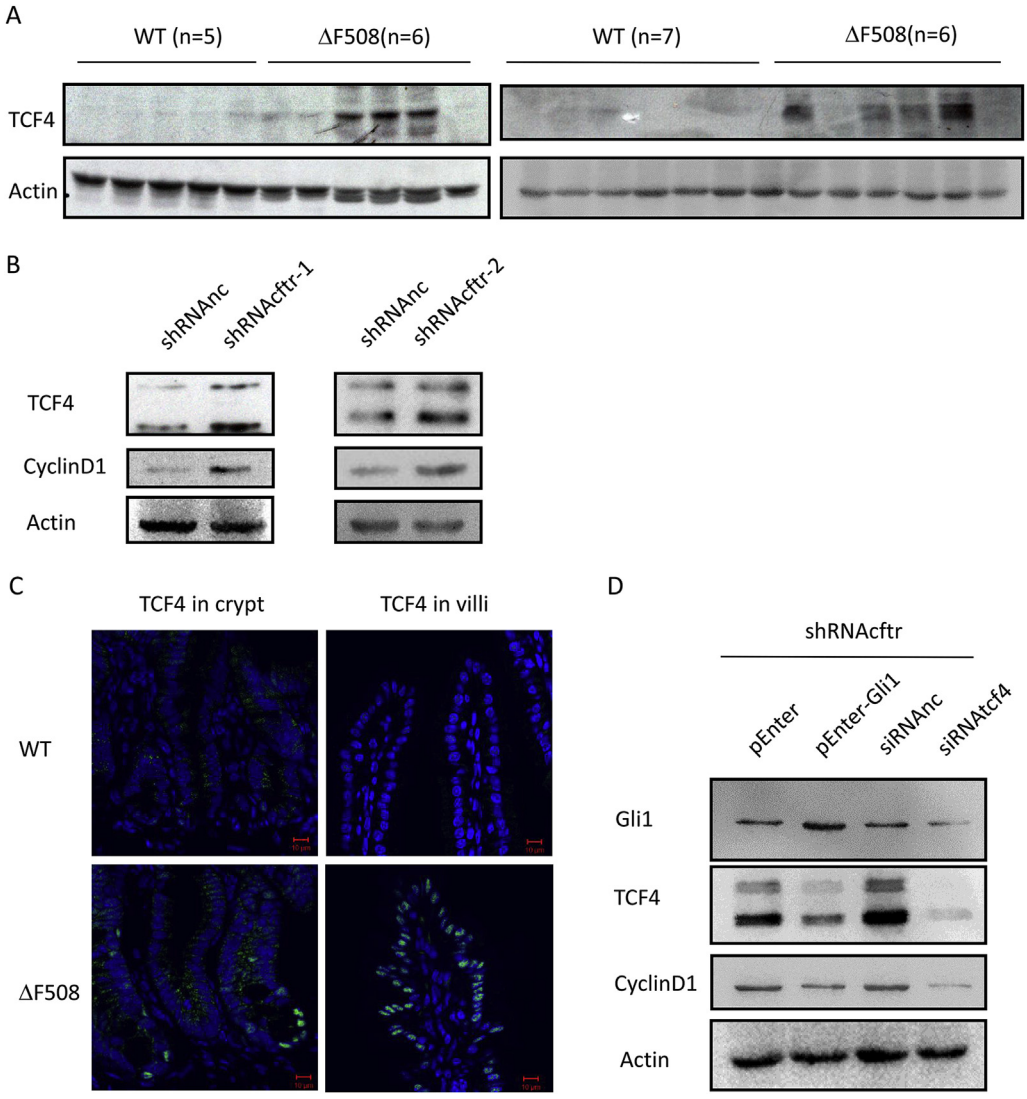

Fig. 5. Expression of TCF4 and CyclinD1 in the ΔF508 mouse small intestine and CFTR-knockdown Caco2 cells.
(A) Western blot analysis of TCF4 in the intestine. Actin was used as a loading control. (B) Western blot analysis of TCF4 and CyclinD1 in CFTR-knockdown Caco2 cells. Actin was used as a loading control. (C) Immunostaining for TCF4 (green; the nucleus was stained with to-Pro3, blue) in the crypt or villi. Scale bar = 10 μm. (D) Western blot analysis of Gli1, TCF4 and CyclinD1 in CFTR-knockdown Caco2 cells with Gli1 overexpression or TCF4 knockdown. Actin was used as a loading control.

3.5. ΔF508 mutation activates TCF4 and Cyclin D1

Gli1 has antagonistic roles in regulating TCF and downstream target genes including CyclinD1 in metastatic colon cancer [28]. Studies have suggested that enhanced Gli1 can attenuate TCF activity and its downstream targets in colon cancer cells [35]. We next analyzed the expression of TCF4 and CyclinD1 in ΔF508 mouse intestines and CFTR-knockdown Caco2 cells by western blotting and immunostaining. Results showed that TCF4 and CyclinD1 were activated significantly (Fig. 5A&B), indicating that proliferation-related pathways were activated. We then performed immunostaining for TCF4 and showed that it was activated in the crypts and villi (Fig. 5C). Overexpression of Gli1 in CFTR knockdown Caco2 cells decreased TCF4 and CyclinD1. Inhibition of TCF4 by siRNA decreased CyclinD1 (Fig. 5D). The results suggest that Gli1 may inhibit TCF4 which regulate CyclinD1.

3.6. Upregulation of β-catenin activates the hedgehog pathway and suppresses proliferation in Caco-2 cells and ΔF508 mouse intestines

We demonstrated that β-catenin and the Hedgehog pathway are downregulated in ΔF508 mouse small intestines and CFTR-knockdown Caco2 cells. We also reported that CFTR stabilizes β-catenin in the small intestine and Caco2 cells [31]. To further investigate whether CFTR regulates Hedgehog signaling through β-catenin, we treated wild-type and ΔF508 mice with LiCl, which can activate β-catenin *in vivo* [36], and analyzed Gli1 expression by western blotting. Results showed that Gli1 downregulation could be reversed by β-catenin activation (Fig. 6A). CFTR-knockdown Caco2 cells were then treated with 10 μM CHIR, which is a β-catenin activator [37], for 24 h and the expression of Gli1 was analyzed by western blotting. Results showed that downregulated Gli1 could be rescued by the CHIR (Fig. 6B), consistent with *in vivo* data. Overexpression of β-catenin in CFTR knockdown Caco2 cells activated Hedgehog pathway (Fig. 6C). In addition, overexpression of β-catenin also upregulated mRNA level of Gli1 in HT29 and HCT116 cells (Fig. 6D). The results implied that the hedgehog pathway can be activated by β-catenin. To clarify the relationship between β-catenin and crypt proliferation, we stained small intestine sections and showed that the increase in PCNA-positive cells in ΔF508 mouse small intestines could be rescued by β-catenin activation (Fig. 6E). These data indicated that decreased β-catenin in the small intestine of CFTR ΔF508

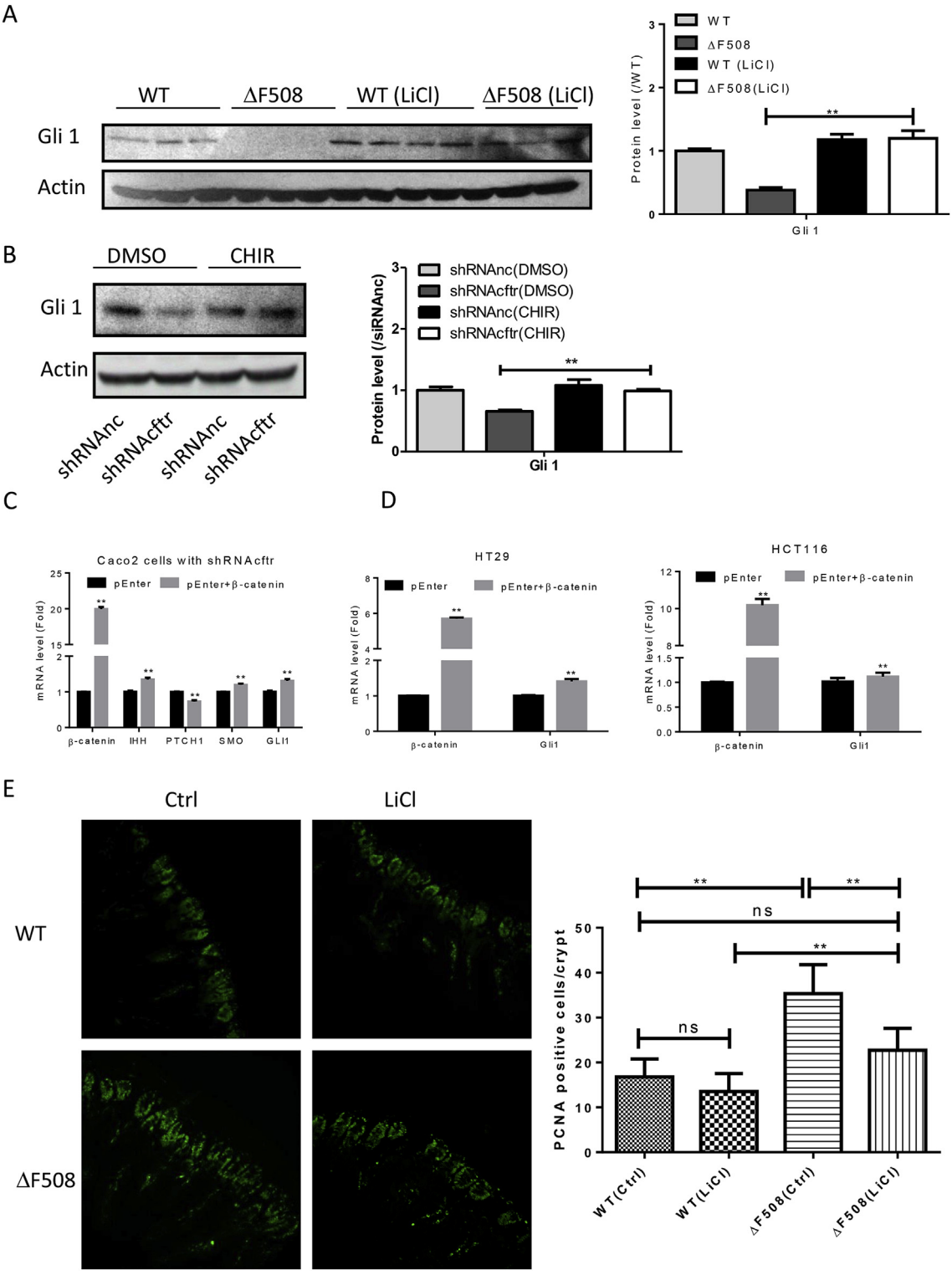

Fig. 6. Relationship between β-catenin and the hedgehog pathway in the ΔF508 mouse intestine and CFTR-knockdown Caco2 cells.
(A) Western blot analysis of the hedgehog pathway with or without LiCl treatment in the small intestine of wild-type and CFTR ΔF508 mice. Wild-type and ΔF508 mice were treated with LiCl, and then Gli1 expression levels were analyzed by western blot. Actin was used as a loading control. The quantification of western blot results is shown in right panel (n = 13). (B) Western blot analysis of the hedgehog pathway with or without CHIR treatment in CFTR-knockdown Caco2 cells. The cells were treated with 10 μM CHIR for 24 h, and the expression level of Gli1 was analyzed by western blotting. Actin was used as a loading control. The quantification of western blot results is shown in the right panel (n = 3). (C) RT-qPCR analysis of Hedgehog pathway in CFTR knockdown Caco2 cells with β-catenin overexpression. (D) RT-qPCR analysis of Gli1 in HT29 and HCT116 cells with β-catenin overexpression. (E) Analysis of PCNA-positive cells in the crypt of the small intestine with or without LiCl treatment. Sections of small intestine were stained with an anti-PCNA antibody (1:200, green). **p < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

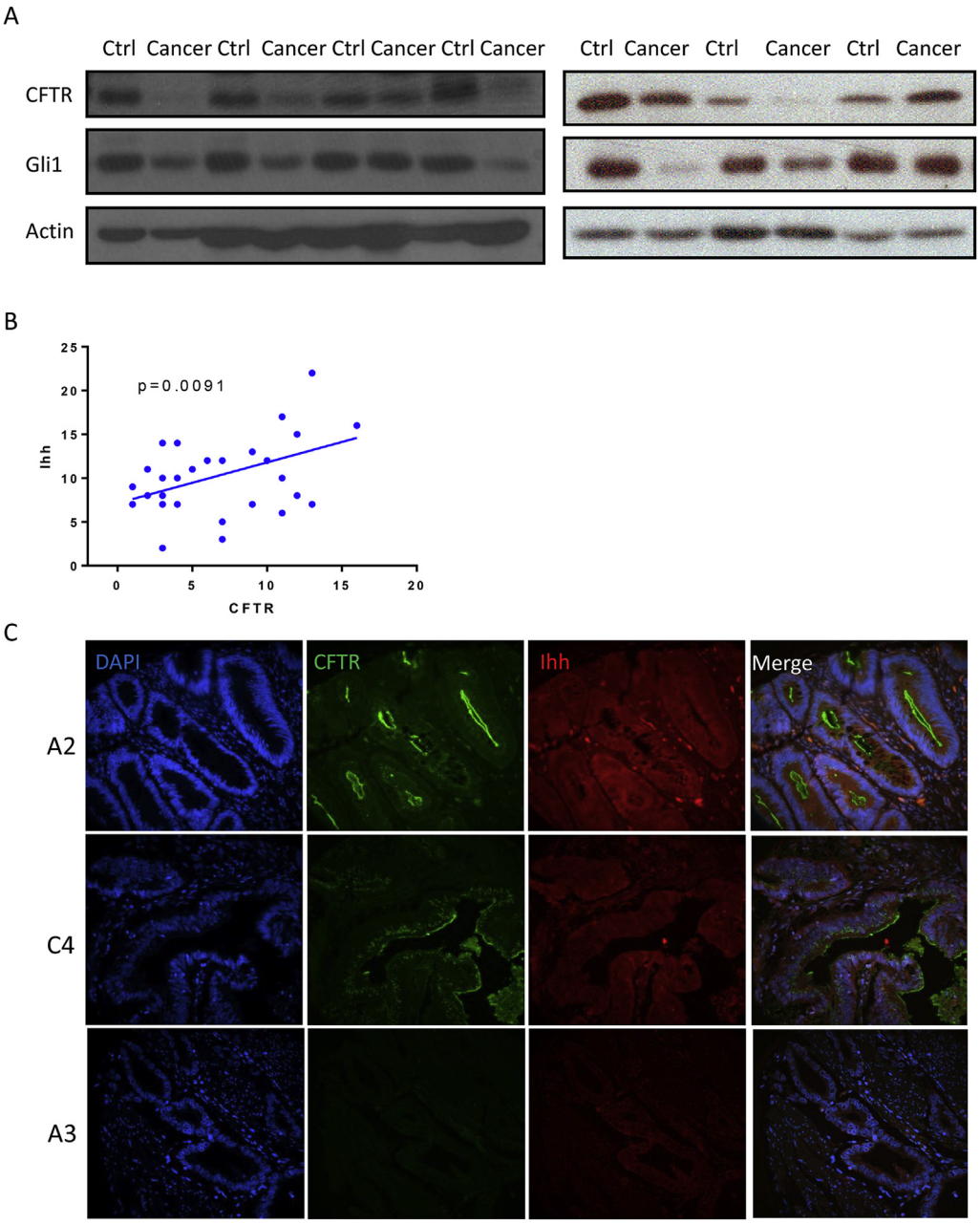

Fig. 7. Expression of CFTR and the Ihh pathway in human colon cancer tissues. (A) Western blot analysis of CFTR and Gli1 in human colon cancer tissues. Actin was used as a loading control. (B) Linear regression analysis of CFTR and Ihh expression in human intestine samples. There was a positive correlation between CFTR and Ihh in a human intestine tissue array. (C) Representative images showing the positive correlation between CFTR and Ihh expression in the human intestine. A2, C4, and A3 represent the positions of the samples on the array. In A2, CFTR was highly expressed at the apical membrane of epithelium cells (green). Ihh was expressed mostly in the epithelial cells. In C4, apical CFTR expression was significantly reduced along with decreased Ihh expression. In A3, CFTR and Ihh expression was remarkably reduced to nearly undetectable levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mice could inhibit hedgehog signaling, which controls intestinal proliferation.

3.7. Identification of strong positive correlation between CFTR and Ihh

It has been shown that *CFTR* mutations induce colon cancer [38] and that CFTR is involved in proliferation in the crypts of the small intestine. To investigate if CFTR is involved in colorectal cancer development, we examined the expression of it and Gli1 in primary tumors and normal tissue by western blotting. Results showed that the expression of CFTR and Gli1 were lost in cancer tissues (Fig. 7A). We also examined CFTR and Ihh expression in human intestinal normal/cancer samples using a tissue array. Results showed a positive correlation between CFTR and Ihh in intestinal normal/cancer tissues (Fig. 7B). Representative images showed the positive correlation between CFTR and Ihh expression in the human intestine. A2, C4, and A3 represent the position of samples on the array. In A2, CFTR was highly expressed at the apical membrane of the epithelium cells (green). Ihh was expressed mostly in epithelial cells. In C4, apical CFTR, along with Ihh, expression was significantly reduced. In A3, CFTR and Ihh expression was remarkably reduced to nearly undetectable levels (Fig. 7C).

4. Discussion

Our results, for the first time, reveal that CFTR regulates Ihh through β-catenin in the mouse intestine and human intestinal epithelial cells. CFTR dysfunction increases the degradation of β-catenin and inhibits the hedgehog pathway, leading to TCF4 and CyclinD1 transcriptional activation, thereby promoting proliferation of the intestinal epithelium (Fig. 8).

Here, we investigated the roles of CFTR in mouse intestinal proliferation. We found that crypt depth was increased significantly in the ΔF508 mouse small intestine, which was accompanied by the marked upregulation of PCNA, indicating activation of proliferation. We further demonstrated that proliferation in the ΔF508 mouse small intestine and

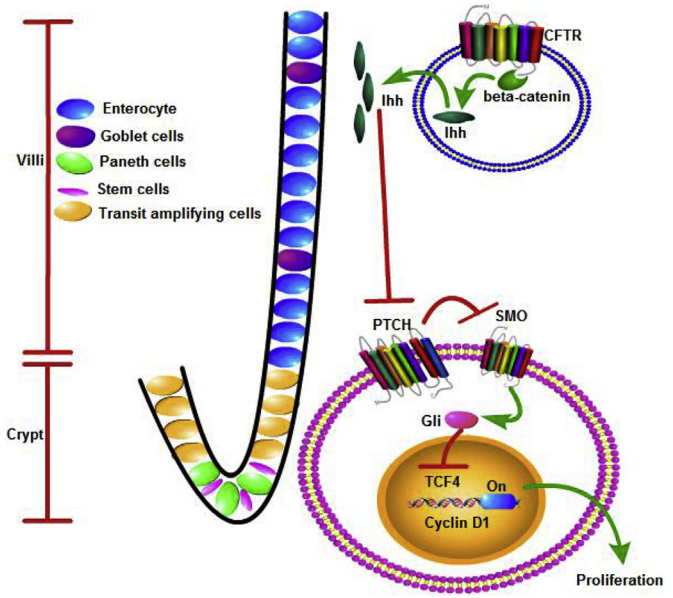

Fig. 8. Schematic illustration of the molecular mechanisms underlying CFTR mutation-induced inhibition of the Hedgehog pathway and activation of the TCF4 transcriptional pathway in the mouse intestine.

CFTR-knockdown Caco2 cells was caused by inhibition of hedgehog signaling due to β-catenin degradation, with disrupted CFTR–β-catenin interactions. The hedgehog pathway, which regulates the proliferation of crypts in the intestine, was markedly downregulated in ΔF508 mouse small intestines and CFTR-knockdown Caco2 cells. We also demonstrated that β-catenin could regulate the hedgehog pathway. Activation of β-catenin *in vitro* and *in vivo* reversed the downregulation of Gli1, which is a key transcription factor of the hedgehog pathway. Importantly, activating β-catenin *in vivo* using LiCl reduced the numbers of proliferating cells in the intestinal crypts of ΔF508 mice.

Recent research has clearly demonstrated that the role of Hedgehog signaling is not restricted to intestinal development, and that it is important for the maintenance of adult small intestine homeostasis [39]. Ihh is produced by the superficial epithelium and signals to the underlying mesenchyme where it targets smooth muscle cells, myofibroblast-like cells, and possibly myeloid cells [19]. Ihh seems to be a key signal emitted by the superficial epithelium to indicate its integrity [40]. Loss of this signal itself is sufficient to trigger not only the activation of an epithelial repair program and the influx of fibroblasts and macrophages, but also mesenchymal immune response activation [26]. It has been reported that undifferentiated Caco2 cells express lower levels of *CFTR* mRNA, whereas a 10-fold increase was observed in differentiated cells [41]. Our data also showed that CFTR was increased with the increase in Ihh after differentiation or became polarized, and that Ihh was downregulated in CFTR-knockdown Caco2 cells (Supplementary Fig. S1). These results suggest that Ihh can be regulated by CFTR during differentiation.

The dramatically reduced expression of CFTR in human colorectal cancer tissues identified herein is consistent with our previous findings using human breast cancer tissues, prostate cancer tissues, and colon cancer tissues, and suggests that the loss of CFTR is likely a general phenomenon in epithelial-derived neoplasms of various organs. Although improved life expectancy among patients with CF has unmasked a significant increase in the incidence of gastrointestinal malignancies based on some cohort studies, the relationship between CFTR and cancer is still controversial. Increased incidence of colorectal cancer was found in CF patients recently. Based only on expression profiles and epidemiological incidence, it is difficult to conclusively determine whether the absence of CFTR is the outcome of tumorigenesis or a driver of cancer development. Identifying the related underlying molecular mechanisms is critical to understand the exact roles of CFTR in various cancers. Conditional knockout of Ihh activates proliferation in the crypt and increases the crypt size in the mouse small intestine [19]. However, how Ihh is regulated requires further investigation. In most colon cancers, Wnt//β-catenin signaling is activated [42]; however, in the small intestine of CFTR ΔF508 mice, the Wnt//β-catenin pathway was inhibited. It has been reported that Wnt-1 and β-catenin production are upregulated in patients with advanced cancer [43]. Interestingly, in human melanoma cancer cells and tissues, β-catenin was downregulated, and treatment using Wnt3a, which can activate Wnt/β-catenin signaling, can inhibit the proliferation of melanoma cells [44]. The present study reveals for the first time that activated proliferation might be regulated by the hedgehog pathway, mediated by β-catenin, which is stabilized by CFTR.

One important question remains after this study. In CFTR ΔF508 mouse small intestines and CFTR-knockdown Caco2 cells, we found that both Hedgehog and Wnt/β-catenin signaling were inhibited. It has been reported that proliferation in the crypts of the small intestine is controlled by Wnt/β-catenin signaling and that the Hedgehog pathway negatively regulates Wnt/β-catenin signaling in colonic epithelial cells during differentiation [25]. Although there are some reports showing that inactivated β-catenin can promote melanoma cell proliferation and that activated β-catenin can inhibit melanoma cell proliferation [44], this is different from the proliferation that occurs in the small intestine. Although proliferation was activated in the crypts of Ihh-mutant mouse small intestines [26], it is still unknown how proliferation is activated. In Ihh-mutant mice, β-catenin is activated [26], but in CFTR ΔF508 mice, it was inhibited, which might have been due to loss of the β-catenin pool; this indicates that there might be other pathways that regulate the proliferation of crypt cells, such as activated TCF4 and CyclinD1, and increased intestinal stem cells (Supplementary Fig. S2), which we also identified. Further works are inspired to determine whether these factors affect the proliferation. It has been reported that Gli1 overexpression inhibits β-catenin/TCF reporter activity and nuclear β-catenin accumulation in human colorectal cancers [45]. Gli1 inhibits TCF in human colon carcinomas [35]. Cyclin D1 is a TCF4 target gene in colorectal cancer [46]. In the CFTR knockdown Caco2 cells, Hedgehog pathway was inhibited, whereas TCF4 and CyclinD1 were activated. Our results implied that downregulation of Gli1 might release the inhibition on TCF and activate CyclinD1 which promotes cell proliferation. This indicates that CFTR is important for the proliferation of the small intestine and that it is controlled by hedgehog pathway-mediated by β-catenin signaling; moreover, cancer risk increases with CFTR mutations, which induces the loss of proliferation control.

Taken together, we demonstrated a previously undefined role for CFTR in regulating epithelial cell proliferation through the regulation of the hedgehog pathway, which is mediated by β-catenin, suggesting its involvement in colorectal cancer development.

Declarations of interest

None.

Conflicts of interest

None declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.12.018.

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