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Original Article

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CTNNAL1 deficiency suppresses CFTR expression in HDM-induced asthma mouse model through ROCK1-CAL signaling pathway

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Abstract

The downregulation of adhesion molecule catenin alpha-like 1 (CTNNAL1) in airway epithelial cells of asthma patients and house dust mite (HDM)-induced asthma animal models was illustrated in our previous study. It is assumed to contribute to airway inflammation and mucus hypersecretion. In this work, we further explore the underlying mechanism of CTNNAL1 in asthma. *CTNNAL1*-silenced female mice exhibit a decreased level of cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated and ATP-gated Cl^- channel that correlates with mucus hypersecretion. Our previous study demonstrated that ROCK1 expression decreases but ROCK2 expression increases in the lungs of a *CTNNAL1*-silenced mouse model. Inhibition of ROCK1 leads to a reduction in CFTR expression in CTNNAL1-overexpressing and *CTNNAL1*-silenced human bronchial epithelial (HBE) cells. It has been reported that ROCK1 is a downstream target of RhoA and that activation of RhoA increases CFTR expression after CTNNAL1 deficiency *in vitro* and *in vivo*. The above results indicate that CTNNAL1 regulates CFTR expression through the ROCK1 pathway. In addition, the expression of CFTR-associated ligand (CAL) is increased after *CTNNAL1* silencing, and immunoprecipitation results confirm the interaction between ROCK1 and CAL. Inhibition of CAL does not influence ROCK1 expression but increases CFTR expression in *CTNNAL1*-silenced HBE cells. These data suggest that CTNNAL1 deficiency decreases CFTR expression in the HDM-induced asthma mouse model through the ROCK1-CAL signaling pathway.

Key words asthma, CTNNAL1, CFTR, ROCK1, CAL

Introduction

Asthma is a consequence of complex gene–environment interactions, with heterogeneity in clinical presentation and the type and intensity of airway inflammation and remodeling [1]. Asthma is a common airway disease characterized by reversible airway obstruction, airway high responsiveness, airway inflammation and increased mucus [2,3]. It is one of the most common chronic diseases and affects approximately 358 million people worldwide. The global prevalence of asthma in adults is approximately 4.3%, with wide variation between countries [1]. The prevalence in developed countries is 20–60 times higher than that in developing countries [4]. However, asthma mortality is high in low- and middle-income countries, where basic asthma medications are not available and patients have difficulties accessing health care [5].

Airway epithelial cells are the first barrier to bacteria, viruses and environmental pollutants [6,7], and the structural damage and loss of functional homeostasis in these cells could aggravate airway inflammation. Our previous study demonstrated that the airway structural adhesion molecule catenin alpha-like 1 (CTNNAL1) was downregulated in asthma patients and an ovalbumin-induced asthma mouse model [8]. CTNNAL1, an 82 kDa protein mapped to 9q312, has similarities to human vinculin and α-catenin, especially in the N-terminal region, which contains binding sites

for β-catenin, talin and α-actinin [9]. There is a negative correlation between the reduction in CTNNAL1 expression and airway hyperresponsiveness [10]. CTNNAL1 deficiency enhances lung inflammation and mucus hypersecretion through activation of the YAP-ROCK2 signaling pathway [11].

It has been demonstrated that CTNNAL1 acts as a scaffold protein for Lbc Rho guanine nucleotide exchange factors (GEFs) and facilitates Lbc-induced Rho signals. Rho family GTPases play important roles during the organization of the actin cytoskeleton and the formation of focal adhesion proteins [12]. Rho-associated protein kinase (ROCK), a serine/threonine kinase, is a downstream target of the GTPase RhoA [13]. Two ROCK isoforms have been identified: ROCK1 (also called ROCK I, ROCK β or p160ROCK) and ROCK2 (also called ROCK II, ROCK α or Rho kinase). *ROCK1* and *ROCK2* are located on chromosomes 18 (18q11.1) and 2 (2p24), respectively. The two ROCK homologues share 64% identity in their primary amino acid sequences, with the highest homology (92%) within the kinase domains and the coiled-coil domains being the most diverse (55%) [14,15]. Studies have shown that ROCK1 and ROCK2 have functional overlaps in some cases; however, they cannot be replaced by each other [14,16,17]. Our previous study demonstrated that ROCK1 expression decreases but ROCK2 expression increases in the lungs of a *CTNNAL1*-silenced mouse model and that CTNNAL1 deficiency increases mucin5AC (MUC5AC) secretion through the ROCK2 signaling pathway [11]. However, it is unknown whether CTNNAL1 regulates the expression of cystic fibrosis transmembrane conductance regulator (CFTR) through the Rho/ROCK signaling pathway.

Mucus consists of water, mucin, ions and so on. MUC5AC and mucin5B (MUC5B) are integral components of airway mucus [18]. In our previous study, we demonstrated that CTNNAL1 deficiency results in an increase in MUC5AC secretion but does not influence MUC5B expression [11]. CFTR is an apical membrane chloride channel critical to the regulation of fluid, chloride, and bicarbonate transport in epithelial and other cell types [19]. CFTR is a multidomain membrane protein that belongs to the large family of adenine nucleotide-binding cassette transporters consisting of two transmembrane domains, two nucleotide binding domains (NBDs) and a unique regulatory domain [19,20]. In human bronchial epithelial (HBE) cells, CFTR functions not only as an anion channel, which conducts Cl^- and bicarbonate [21] but also as a regulator of the amiloride-sensitive epithelial Na^+ channel (ENaC) that constitutes the limiting pathway for Na^+ absorption [20]. CFTR mutation or deficiency leads to an imbalance in salt and fluid transport that results in dehydrated and viscous secretions.

It has been reported that CFTR-associated ligand (CAL) could bind with CFTR, and overexpression of CAL in cells could lead to a reduction in the plasma-membrane levels of CFTR [22]. The negative effect of CAL overexpression on CFTR expression levels could be reversed by overexpression of TC10, a member of the Rho family, whose constitutively active form redistributes intracellular CFTR towards the plasma membrane [23]. However, whether CTNNAL1 deficiency suppresses CFTR expression through CAL signaling pathway remains unknown.

In this study, we intend to duplicate the CTNNAL1-deficient mouse model and human bronchial epithelial cell model and further investigate the function and mechanism of CTNNAL1 in mucus hypersecretion and CFTR expression upon HDM exposure.

Material and Methods

Ethics statement

The study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal study was approved by the Xiangya Animal Protection and Use Committee of Central South University (No. 2020sydw0305). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering of the animals.

Animals and challenge protocols

Animal experiments were conducted on 6-week-old female c57bl/6j mice which were obtained from the experimental animal center, Xiangya School of Medicine, Central South University and housed under barrier conditions in air-filtered, temperature-controlled units under a 12/12 h light/dark cycle and with free access to food and water. AAV5-CTNNAL1-RNAi mice and AAV5-CON305 mice were established as described previously [11]. Following transfection, an HDM-stressed asthma model was constructed according to a previous publication [24]. The mice were sensitized to purified HDM extract (10 μg protein in 50 μL saline; Greer Laboratories, Lenoir, USA) intranasally (i.n.) on days 14, 21, 28 and 34. The allergen challenge was performed via atomization treatment of HDM extract on four consecutive days (days 35–38), and the mice were sacrificed 24 h after the last challenge (day 39). The control group received an equal volume of sterile PBS via i.n. route and atomization route on days 14, 21, 28, 34, 35, 36, 37 and 38.

Cell culture, transfection, and treatment

Human bronchial epithelial (16HBE14o-) cell line was purchased from Lifeline Cell Technology (Frederick, USA). HBE cells were cultured in DMEM (Sigma-Aldrich, St Louis, USA) with 50 U/mL penicillin, 50 U/mL streptomycin, 10% foetal bovine serum (Sigma-Aldrich) and incubated at 37°C in 5% CO_2. Then 50 nM CTNNAL1 siRNA (5′-GGCTACCTTTCACTTCCAA-3′) or nonsense siRNA (stB000334) from RiboBio (Guangzhou, China) was transfected into HBE cells using Lipofectamine 3000 (Invitrogen, Carlsbad, USA) for 24 h. After transfection, HBE cells were incubated with HDM for 24 h. In the inhibitor treatment group, cells were pretreated for 30 min with Y27632 (10 μM, ROCK1 inhibitor; MedChem Express, Monmouth Junction, USA) or KD025 (40 μM, ROCK2 inhibitor; MedChem Express) before incubation with HDM extract. In the agonist treatment group, cells were pretreated for 30 min with CN03 (1 μM, Rho agonist; MedChem Express) before incubation with HDM extract.

Overexpression plasmid transfection

The effective CTNNAL1 plasmid (5′-GTTCTATAATTACGAACAA-3′) was designed and synthesized by Genechem (Shanghai, China). Transfections were conducted using Lipofectamine 3000 and p3000 (Invitrogen) in accordance with the manufacturer's instructions.

Trachea sampling and treatment

Mouse tracheae were taken from the AAV5-CON305 or AAV5-CTNNAL1-RNAi mouse groups. Mouse tracheae were cultured in serum-free medium, and after 24 h of culture, HDM and CN03 (1 μM) were applied to the culture system.

Immunohistochemistry

Paraffin-embedded lung tissue sections were deparaffinized, rehydrated, immersed in 3% hydrogen peroxide for 10 min, and then incubated for 30 min in blocking buffer (5% bovine serum albumin). The sections were incubated overnight at 4°C with antibodies against CTNNAL1 (1:200; Abcam, Cambridge, UK) or CFTR (1:200; Bioss, Beijing, China), followed by incubation at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:100; Sigma-Aldrich). After color development, images were acquired with a BA410E microscope (Motic, Xiamen, China).

Hematoxylin-eosin (HE) staining

Paraffin-embedded sections were sliced at a thickness of 3 μm and then stained using hematoxylin and eosin (HE; Sigma-Aldrich). Different parameters in sections of lung tissue were measured and used to assess the degrees of lung injury, grading four histological observations: hemorrhage, alveolar congestion, filtration or aggregation of neutrophils in the airspace or vessel wall, and thickness of the alveolar wall/hyaline membrane formation. Next, lung damage was scored using a four-point scale: none (0), mild (1), moderate (2), and severe (3) lung injury by three pathologists blinded to the experimental conditions [25]. The scores from all three were averaged to give a final score.

Scanning electron microscopy

Mouse tracheae were taken from the AAV5-CON305 and AAV5-CTNNAL1-RNAi mouse groups. Subsequently, the tissues were fixed with cold (4°C) 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After 1 h of fixation, the samples were trimmed and then transferred to fresh fixative at 4°C for another 3 h. The samples were washed with cold (4°C) dilute buffer (equal volume of 0.1 M sodium cacodylate buffer and distilled water); dehydrated in cold conditions (4°C) with graded ethyl alcohol in ascending concentrations, ethyl alcohol and acetone in the ratios 3:1, 1:1 and 1:3 and with anhydrous acetone, and dried using a Critical Point Dryer (E3000 series; Quorum Technologies, East Sussex, UK) with liquid carbon dioxide as the transitional fluid. The tissues were then attached to stubs, coated with gold using an SC7620 Sputter Coater (Quorum Technologies) and examined with an EVO LS 10 Scanning Electron Microscope (Carl Zeiss, Oberkochen, Germany).

Immunofluorescence staining

HBE cell slides were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min and then washed three times with PBS. Slides and cells were blocked with 1% bovine serum albumin for 30 min at room temperature. Then, the cells were incubated with an antibody against CFTR (1:100; Bioss) overnight at 4°C, followed by staining with the corresponding secondary antibodies at room temperature (1:200). 4′,6-Diamidino-2-phenylindole (DAPI; Solarbio, Beijing, China) was used to stain the cell nuclei. Images were visualized using an Axioscope 5 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Western blot analysis

Total protein lysates were extracted from the lung tissues of mice or HBE cells using RIPA lysis buffer (Sigma-Aldrich) supplemented with proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). The total protein concentration was measured using a BCA kit (Thermo Fisher Scientific, Waltham, USA). The total proteins were separated by 10% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Billerica, USA). After being blocked in 5% fat-free milk for 2 h, the membranes were incubated with primary anti-GAPDH (Ab9485; 1:1000; Abcam), anti-CTNNAL1 (Ab96184; 1:1000; Abcam), anti-ROCK1 (Ab134181; 1:1000; Abcam), anti-CFTR (AF7719; 1:1000; Affinity, London, UK), and anti-CAL (Ab109119; 1:1000; Abcam) overnight at 4°C. The corresponding horseradish peroxidase (HRP) secondary antibody (Ab288151; 1:7500; Abcam) was applied for 1 h at room temperature. Immunoreactive bands were detected with enhanced chemiluminescence reagents (Millipore) on the Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, USA). The abundance of the targeted protein was analyzed using Image Lab analysis software by normalizing to the protein level of GAPDH. All experiments were performed at least three times.

Quantitative RT-PCR

RNAiso Plus (Takara Clontech, Kyoto, Japan) was used to extract total RNA from lung tissues or HBE cells. After quantification using an ultraviolet spectrophotometer (Thermo Fisher Scientific), 1 μg of total RNA was used to synthesize complementary DNA using the Prime Script™ RT reagent kit with genomic DNA Eraser (Takara Clontech). Quantitative real-time PCR analyses were conducted using the SYBR® Premix Ex Taq™ II system (Takara Clontech) on a deep well Real-Time PCR Detection System (CFX96 Touch™; Bio-Rad). The PCR cycling conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 15 s, and 60°C for 30 s. *GAPDH* was used as the internal control. Each experiment was performed in triplicate in three replicate wells. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [8]. Primer sequences of the mouse and human genes used in this study are shown in Tables 1 and 2.

Statistical analysis

All experiments were performed at least in triplicate, and analysis was performed using statistical software PRISM (GraphPad, La Jolla, USA). An unpaired *t* test was used for comparisons between two groups. Differences among multiple groups were determined by ANOVA, followed by Bonferroni correction for multiple comparison testing. Data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

CTNNAL1-deficient mouse model was successfully constructed

AAV5-CTNNAL1-RNAi was used to silence *CTNNAL1* in broncho-

Table 1. Primer sequences of mouse genes for RT-qPCR

Gene	Primer sequence
CTNNAL1	Forward: 5′-AGATGAGTGACATGGCGACG-3′
	Reverse: 5′-CAGCCCGAGCTTTGCTATCT′
CFTR	Forward: 5′-TCGCTGGTTGCACAGTC-3′
	Reverse: 5′-TGCCTGAAGGGAGTCGT-3′
ROCK1	Forward: 5′-GTGGTATTGAAAGCCGCACTG-3′
	Reverse: 5′-TGCCATCTATTCATTCCAGCCAT-3′
GAPDH	Forward: 5′-TTGCAGCTCCTTCGTTGCC-3′
	Reverse: 5′-GACCCATTCCCACCATCACA-3′

Table 2. Primer sequence of human genes for RT-qPCR

Gene	Primer sequence
CTNNAL1	Forward: 5′-CTACACCAGCCATGAGCA-3′
	Reverse: 5′-GCCTGAGTTGACAGTTCCA-3′
CFTR	Forward: 5′-CCAAACCTCACAGCAACTC-3′
	Reverse: 5′-AAGGCACGAAGTGTCCATA-3′
CAL	Forward: 5′-GCCCTCAAACCTCTCTTCC-3′
	Reverse: 5′-CAACGGCTTTCTTCTCCCT-3′
ROCK1	Forward: 5′-CAAACGATATGGCTGGAAG-3′
	Reverse: 5′-TGGATTGGATTGCTCCTTA-3′
GAPDH	Forward: 5′-GACGCTCACCCCAGACA-3′
	Reverse: 5′-TGACACCCCACAGCAAGA-3′

pulmonary tissues of mice. CTNNAL1 mRNA expression was detected by RT-qPCR at 2, 4, 6, and 8 weeks after AAV5-CTNNAL1-RNAi or AAV5-CON305 injection (Supplementary Figure S1A). At 2 weeks post-injection, CTNNAL1 mRNA expression in the lung tissues of mice in the AAV5-CTNNAL1-RNAi group was 25.31% lower than that in the AAV5-CON305 group. At 4 weeks and 6 weeks post-injection, CTNNAL1 mRNA expression significantly decreased. At 4 weeks post-injection, CTNNAL1 protein expression and mRNA expression decreased by approximately 75% and 65%, respectively, compared with those in the control group and AAV5-CON305 group (Supplementary Figure S1B–D). The silencing efficiency of CTNNAL1 at 4 and 6 weeks post-injection was greater than that at 2 and 8 weeks post-injection. Frozen sections of lung tissues showed strong fluorescence in the lung tissues of mice after adeno-associated virus (AAV) infection, indicating that AAV successfully infected bronchopulmonary tissues (Supplementary Figure S1E). The immunohistochemistry results showed that CTNNAL1 expression was significantly decreased in the AAV-CTNNAL1-RNAi group compared with that in the AAV5-CON305 group (Supplementary Figure S1F,G). These data indicated that CTNNAL1-deficient mouse model was constructed successfully.

CTNNAL1 deficiency facilitated airway inflammation and mucus hypersecretion in an HDM-induced asthma mouse model

Both HDM stimulation and CTNNAL1 deficiency increased inflammatory cell infiltration into lung tissues. There was marked infiltration of inflammatory cells into the lungs of the AAV5-CTNNAL1-RNAi + HDM group compared with the AAV5-CON305 + HDM group (Figure 1A,B). The histological changes in the tracheae were examined by H&E staining. CTNNAL1 deficiency and HDM stimulation both damaged the tracheal wall, with mild hyperplasia of submucosal glands and collapse, loss and disorder of cilia (represented by black arrow). Furthermore, more serious damage to the tracheal wall, with severe hyperplasia of submucosal

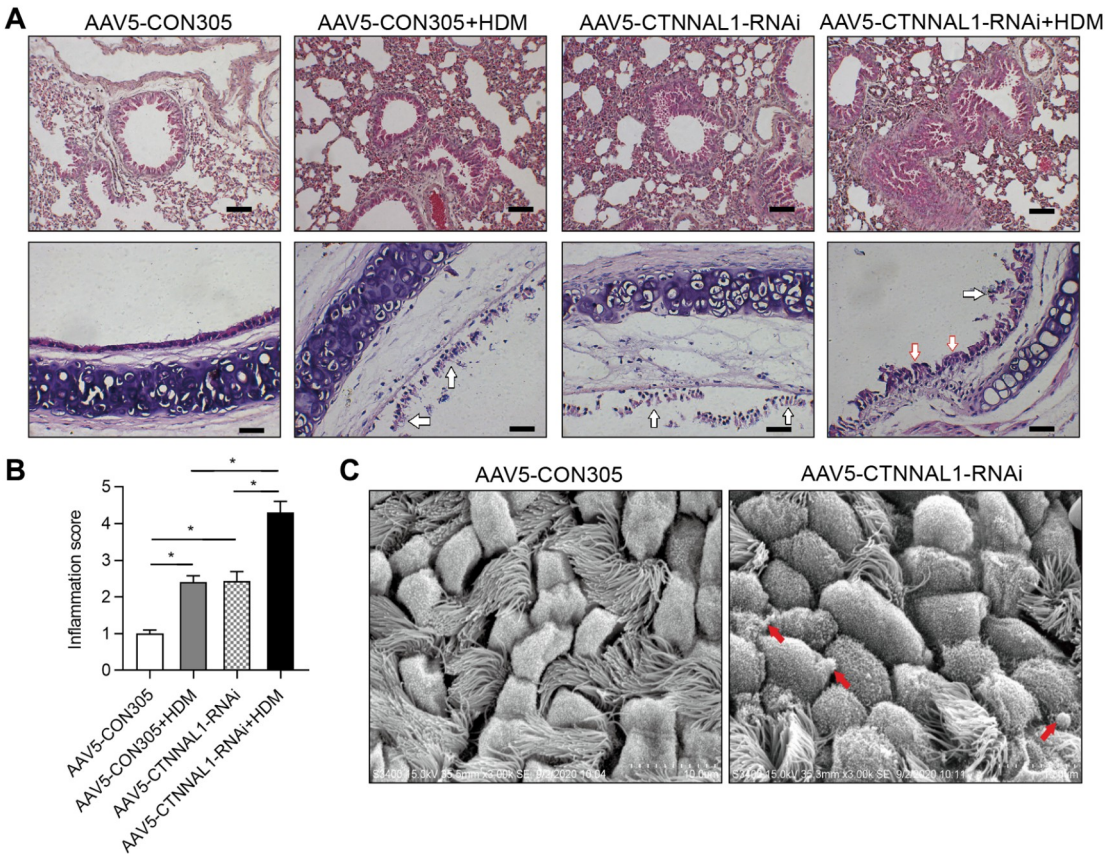

Figure 1. CTNNAL1 deficiency promoted airway inflammation and mucus secretion in the HDM-induced asthma mouse model (A) Lung sections were stained with H&E ($n = 5$; scale bar: 100 μm). Tracheal sections were stained with H&E ($n = 5$; scale bar: 50 μm). (B) The inflammation score was measured independently by three pathologists blinded to the experiment ($n = 5$). (C) Ciliated tracheae were analyzed by scanning electron microscopy ($n = 4$; sacle bar: 10 μm). The experiments were performed three times, and data are presented as the mean ± SD. HDM: 10 μg protein in 50 μL saline. *$P < 0.05$.

glands (represented by red arrow) and loss and disorder of cilia (represented by black arrow), appeared in HDM-treated CTNNAL1-deficient mice than in the HDM-stressed AAV5-CON305 group (Figure 1A). Scanning electron microscopy results showed mucus overproduction and the adhesion and lodging of cilia in the tracheae of the AAV5-CTNNAL1-RNAi mouse group (Figure 1C). These data indicated that CTNNAL1 deficiency promoted airway inflammation and mucus hypersecretion in HDM-treated mice.

CTNNAL1 deficiency decreased CFTR expression in HDM-treated mice

Our previous study illustrated that CTNNAL1 deficiency enhanced mucus hypersecretion and increased MUC5AC expression upon HDM stimulation [11]. Mucus, a gel, consists of water, ions, proteins and macromolecules [26–28]. The major macromolecule components of mucus are mucin glycoproteins, which are critical for local defense of the airway [29]. CFTR is located at the apical membrane of airway epithelial cells and mediates the transport of HCO_3^- and Cl^-, accompanied by water. Dysfunction of CFTR leads to an imbalance in salt and fluid transport that results in dehydrated and viscous secretions [30]. Our results demonstrated that HDM stimulation and CTNNAL1 deficiency both reduced the expression of total CFTR and surface CFTR. Moreover, total CFTR and surface CFTR levels in the AAV5-CTNNAL1-RNAi + HDM group were lower than those in the AAV5-CON305 + HDM group (Figure 2A–C). Immunohistochemistry of CFTR showed its expression in the superficial epithelium in airways. The expression of CFTR in the AAV5-CTNNAL1-RNAi and AAV5-CON305 + HDM mouse groups was lower than that in the AAV5-CON305 group, and it was significantly decreased in the HDM-treated CTNNAL1-deficient group (Figure 2D,E). These data indicated that CTNNAL1 deficiency decreased CFTR expression in HDM-stressed mice.

CTNNAL1 deficiency decreased CFTR expression in HDM-treated HBE cells

To further verify that CTNNAL1 deficiency could lead to the downregulation of CFTR expression *in vitro*, HBE cells were transfected with CTNNAL1 small interfering RNA (siRNA). CTNNAL1 deficiency and HDM stimulation both decreased the expression of CFTR. In addition, the expression of CFTR was significantly decreased in CTNNAL1-silenced HBE cells upon HDM stimulation (Figure 3). These data indicated that CTNNAL1 deficiency decreased CFTR expression in HDM-treated HBE cells.

Inhibition of ROCK1 decreased CFTR expression, but inhibition of ROCK2 did not alter CFTR expression in response to CTNNAL1 deficiency

CTNNAL1 was identified as a part of the Rho signaling pathway [12]. Our previous study demonstrated that CTNNAL1 deficiency decreased ROCK1 expression but increased ROCK2 expression [11]. To investigate whether ROCK1 contributes to CFTR expression when *CTNNAL1* is silenced, we used the ROCK1 siRNA to treat HBE cells. ROCK1 siRNA administration decreased the expression of CFTR in *CTNNAL1*-silenced HBE cells. However, ROCK1 siRNA treatment did not influence CFTR expression in *CTNNAL1*-silenced HBE cells upon HDM stimulation (Figure 4A–C). To investigate

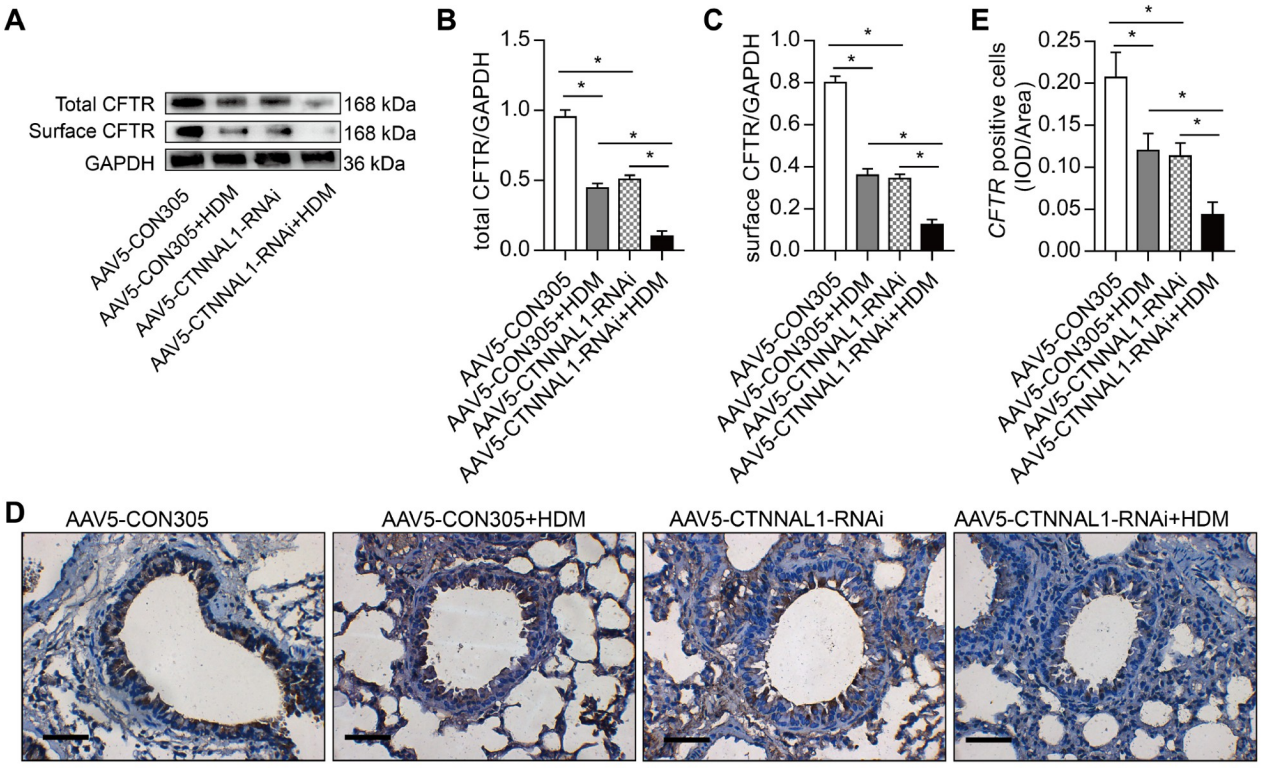

Figure 2. CTNNAL1 deficiency decreased CFTR expression in HDM-treated mice (A–C) Total CFTR and surface CFTR protein expression in the lungs were examined by western blot analysis. GAPDH was used as a loading control ($n = 4$). (D,E) CFTR expression in the airways of mice was detected by immunohistochemistry ($n = 4$; scale bar: 50 μm). The experiments were performed three times, and data are presented as the mean ± SD. HDM: 10 μg protein in 50 μL saline. *$P < 0.05$.

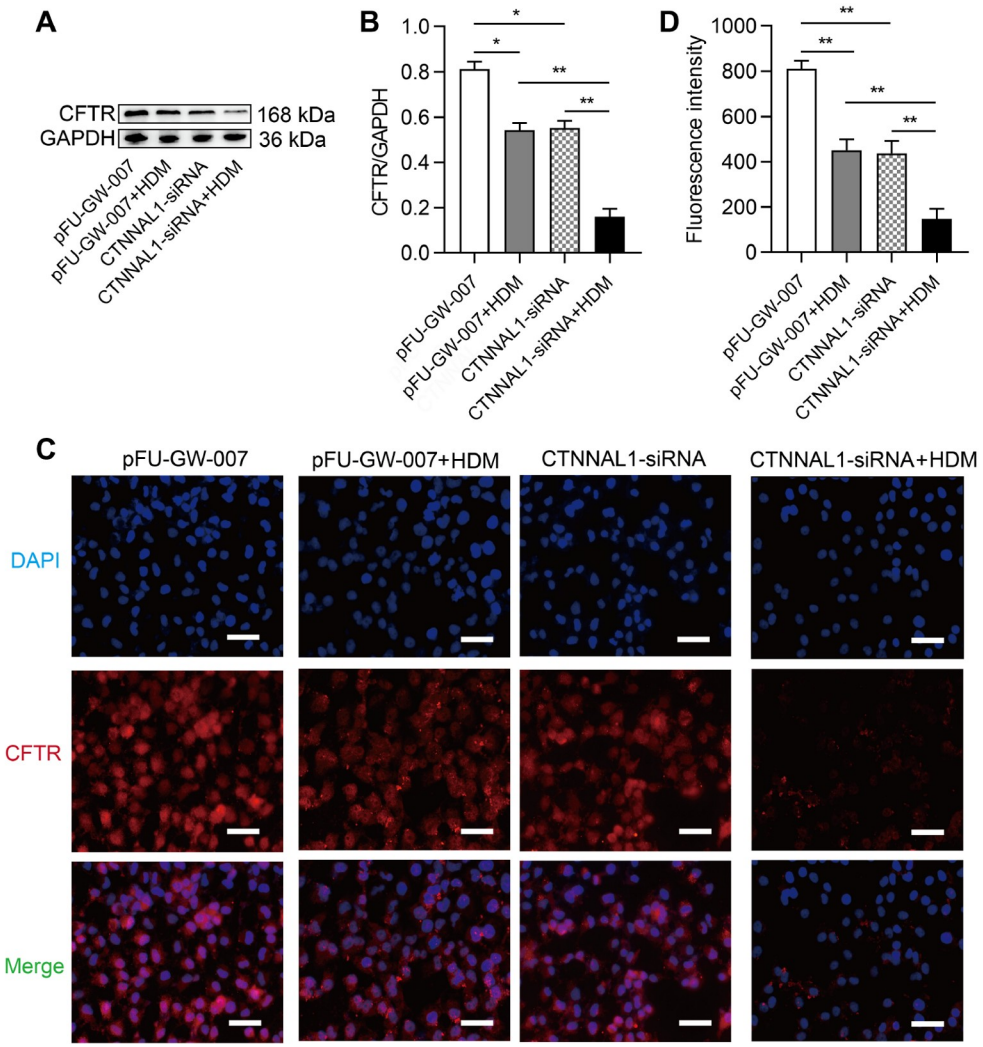

Figure 3. CTNNAL1 deficiency decreased CFTR expression in HDM-treated HBE cells (A,B) CFTR protein expression in HBE cells was detected by western blot analysis ($n=5$). (C) The level of CFTR was assessed by immunofluorescence in HBE cells ($n=3$; scale bar: 50 μm). (D) Immunofluorescence was quantified by the fluorescence intensity ($n=3$). The experiments were performed three times, and data are presented as the mean ± SD; HDM: 10 μg protein in 50 μL saline, pFU-GW-007 was used as control, $^{*}P<0.05$, $^{**}P<0.01$.

whether CTNNAL1 deficiency leads to a reduction in CFTR expression through the ROCK2 signaling pathway, the ROCK2-specific inhibitor KD025 was used to treat CTNNAL1-deficient HBE cells. The results showed that inhibition of ROCK2 did not influence CFTR expression in CTNNAL1-deficient HBE cells (Figure 4D–F). These data indicated that ROCK1 inhibition decreased CFTR expression, but ROCK2 inhibition did not alter CFTR expression in response to CTNNAL1 deficiency.

ROCK1 inhibition decreased CFTR expression in CTNNAL1-overexpressing HBE cells

The expression level of CFTR in HDM-treated *CTNNAL1*-silenced HBE cells was too low to detect the effect of a ROCK1 inhibitor on CFTR expression. To further verify that the ROCK1 signaling pathway participates in CTNNAL1-mediated upregulation of CFTR expression, ROCK1 siRNA was used to treat CTNNAL1-overexpressing HBE cells. A significant decrease in CFTR expression was observed upon HDM stimulation in HBE cells. However, CFTR expression was significantly increased when CTNNAL1 was overexpressed, and HDM stimulation decreased its expression in CTNNAL1-overexpressing HBE cells (Figure 5A,B). The results showed that the protein and mRNA expression levels of CFTR and ROCK1 were significantly decreased after transfection with ROCK1 siRNA (Figure 5A–E). These data indicated that ROCK1 inhibition decreased CFTR expression in CTNNAL1-overexpressing HBE cells.

Activation of RhoA increased CFTR expression in response to CTNNAL1 deficiency

Next, to further verify the involvement of the ROCK1 pathway in the regulation of CFTR expression, tracheae were taken from mice and treated with the RhoA agonist CN03. Remarkably, treatment with CN03 significantly increased the expression levels of CFTR and ROCK1 in *CTNNAL1*-silenced mice with or without HDM stimulation (Figure 6). Meanwhile, the expressions of CFTR and ROCK1 were detected in CTNNAL1-deficient HBE cells upon CN03 stimulation. Administration of CN03 increased the expression levels of CFTR and ROCK1 in CTNNAL1-deficient HBE cells. More importantly, agonist treatment significantly increased their expres-

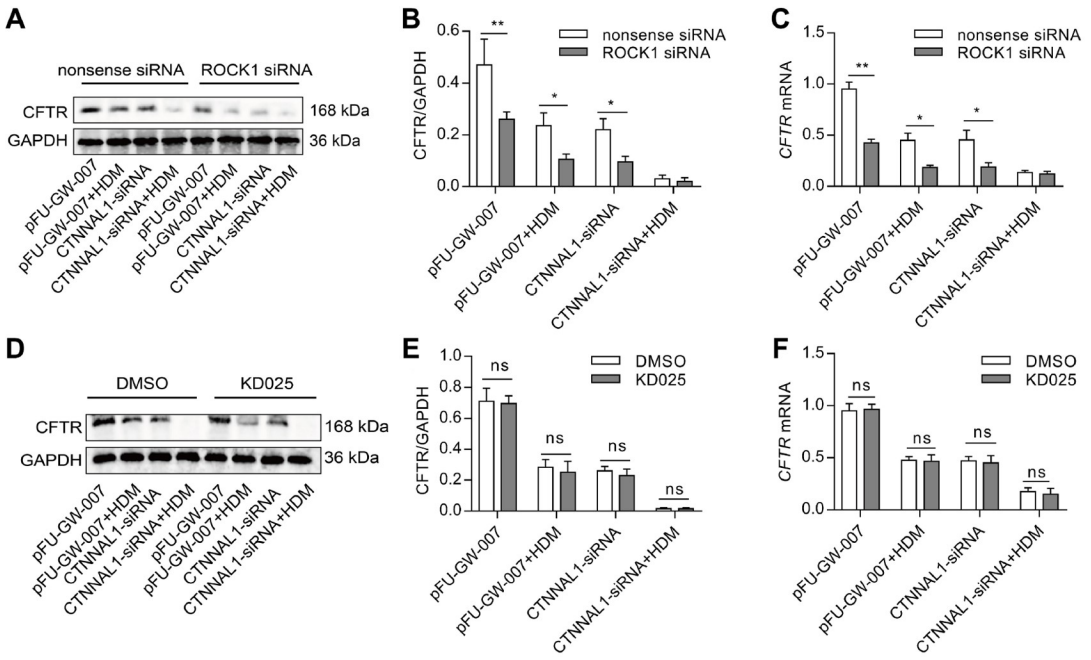

Figure 4. Inhibition of ROCK1 decreased CFTR expression, but inhibition of ROCK2 did not alter CFTR expression in response to CTNNAL1 deficiency (A, B) Protein expression of CFTR in 16HBE14o- cells was detected by western blot analysis ($n=4$). (C) mRNA expression of CFTR in 16HBE14o- cells was detected by RT-qPCR ($n=4$). (D,E) Protein expression of CFTR in 16HBE14o- cells was detected by western blot analysis ($n=4$). (F) mRNA expression of CFTR in 16HBE14o- cells was detected by RT-qPCR ($n=4$). The experiments were performed three times, and data are presented as the mean ± SD; HDM: 10 μg protein in 50 μL saline, KD025: 1 μM, pFU-GW-007 was used as control. $^{*}P<0.05$, $^{**}P<0.01$. ns: no significance

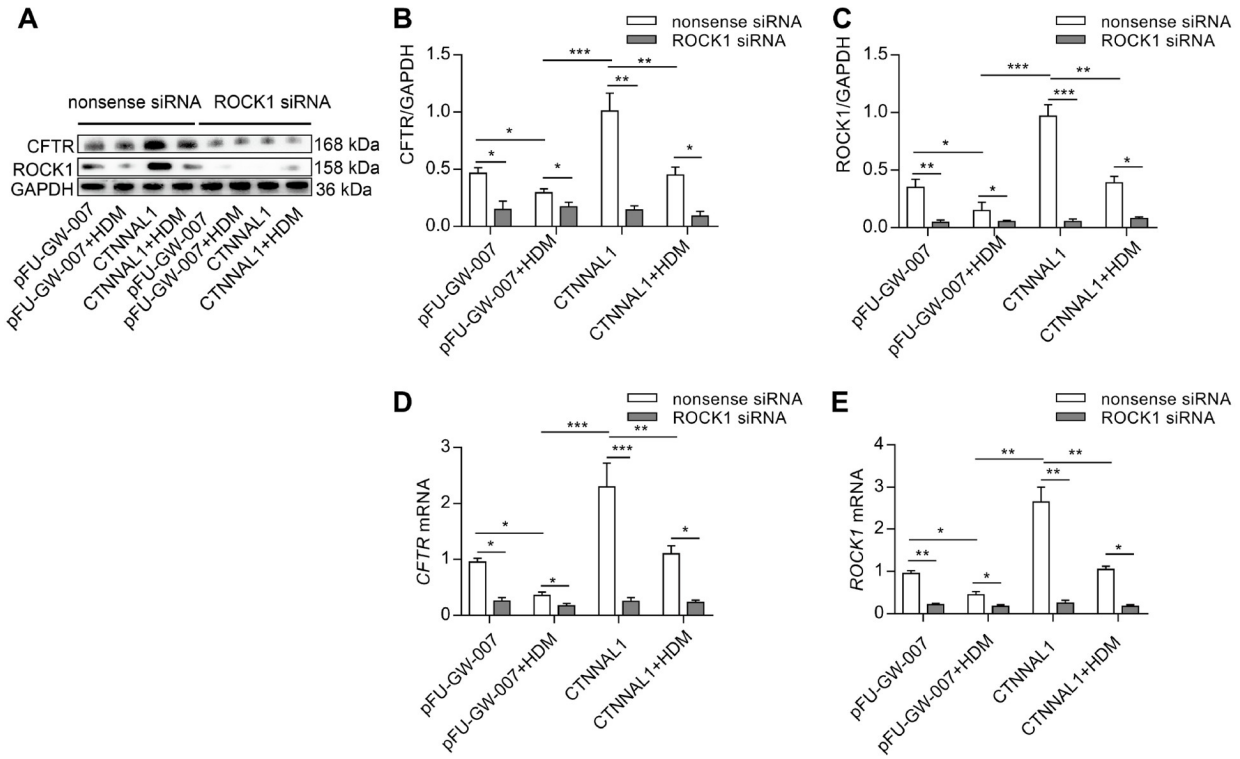

Figure 5. ROCK1 inhibition decreased CFTR expression in CTNNAL1-overexpressing HBE cells (A-C) Protein expression of CFTR and ROCK1 in CTNNAL1-overexpressing HBE cells was detected by western blot analysis ($n=3$). (D, E) mRNA expression of CFTR and ROCK1 in CTNNAL1-overexpressing HBE cells was detected by RT-qPCR ($n=3$). The experiments were performed three times, and data are presented as the mean ± SD; HDM: 10 μg protein in 50 μL saline. pFU-GW-007 was used as control, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

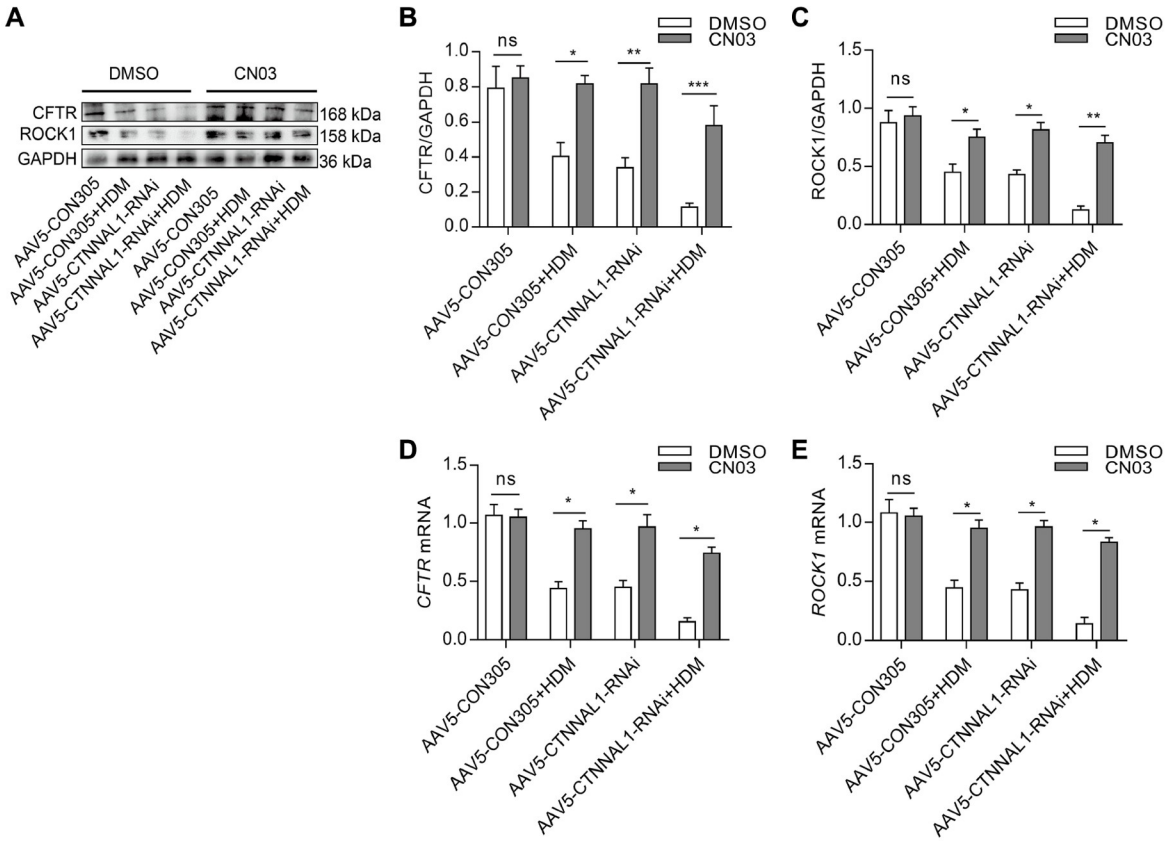

Figure 6. Activation of RhoA increased CFTR expression in *CTNNAL1*-silenced mice (A–C) Protein expression of CFTR and ROCK1 in the tracheae was examined by western blot analysis ($n=3$). (D E) mRNA expression of CFTR and ROCK1 in the tracheae was examined by RT-qPCR ($n=3$). The experiments were performed three times, and data are presented as the mean ± SD; HDM: 10 μg protein in 50 μL saline; CN03: 1 μM. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. ns: no significance.

sions in CTNNAL1-deficient HBE cells after HDM stimulation (Figure 7A–E). Administration of Y27632 decreased the expression level of CFTR in *CTNNAL1*-silenced HBE cells upon CN03 stimulation (Figure 7F–G). These data indicated that RhoA activation increased CFTR expression in response to CTNNAL1 deficiency.

CTNNAL1 deficiency increased the expression of CAL in HBE cells

It has been reported that CFTR-associated ligand (CAL) decreases total and cell surface CFTR by targeting CFTR for degradation in the lysosome [22,31]. Furthermore, the Rho family small GTPase TC10 interacts with CAL. This interaction specifically upregulates CFTR protein expression [23]. To confirm the association between CAL and CTNNAL1, we examined CAL expression in *CTNNAL1*-silenced HBE cells. Our results demonstrated that HDM stimulation and CTNNAL1 deficiency both increased CAL expression *in vitro*. In addition, CAL expression in the HDM-treated CTNNAL1-siRNA group was higher than that in the other groups (Figure 8A–C). These data indicated that CTNNAL1 deficiency increased CAL expression in HBE cells.

CAL inhibition upregulated CFTR expression in response to CTNNAL1 deficiency

To further verify the involvement of CAL in CTNNAL1-regulated CFTR production, we silenced *CAL* by siRNA transfection. It increased the expression of CFTR in the CTNNAL1-siRNA group and pFU-GW-007 + HDM group (Figure 9A,C,F). However, inhibition of CAL did not alter ROCK1 expression in *CTNNAL1*-silenced HBE cells (Figure 9A,B,E). The interaction between CAL and ROCK1 within airway epithelial cells was detected via immunoprecipitation (Figure 9H,I). These data indicated that CAL inhibition upregualted CFTR expression in response to CTNNAL1 deficiency.

Discussion

In the present study, we demonstrated that CTNNAL1 deficiency and HDM stimulation both stimulated inflammatory cell infiltration into lung tissues and damaged the tracheal wall, with mild hyperplasia of submucosal glands and collapse, loss and disorder of cilia. Upon HDM stimulation, CTNNAL1-deficient mice had more severe airway inflammation. Furthermore, CTNNAL1 deficiency led to mucus overproduction and the adhesion and lodging of cilia in the tracheae. In particular, the expression of CFTR was decreased in response to CTNNAL1 deficiency and significantly decreased in CTNNAL1-deficient mice upon HDM stimulation.

CFTR is responsible for cystic fibrosis. It is a multidomain membrane protein that belongs to the large family of adenine nucleotide-binding cassette transporters consisting of two transmembrane domains, two nucleotide binding domains (NBDs) and a unique regulatory domain [19,32]. It is mainly expressed in the airway surface epithelium, submucosal glands and other epithelial organs. CFTR functions as an anion channel and transports Cl^- and

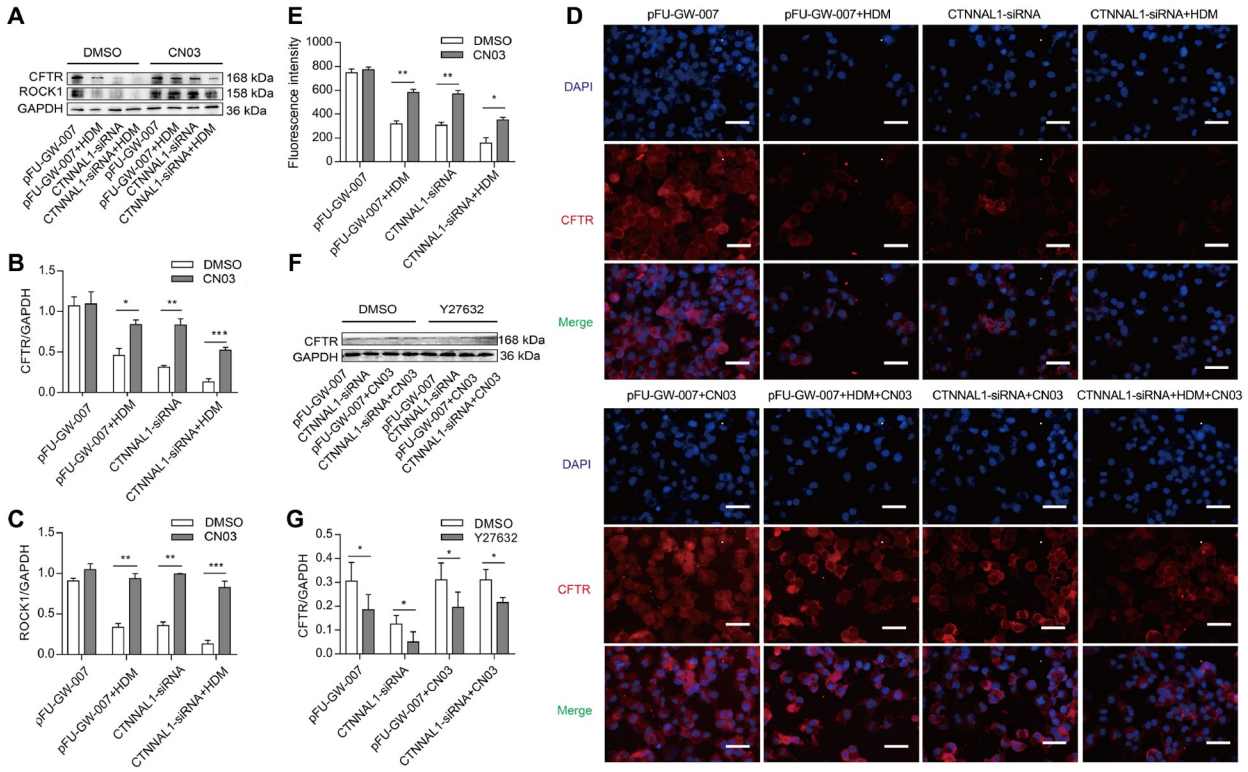

Figure 7. Activation of RhoA increased CFTR expression in *CTNNAL1*-silenced HBE cells (A–C) Protein expression of CFTR and ROCK1 in 16HBE14o- cells was examined by western blot analysis ($n=4$). (D) The level of CFTR in 16HBE14o- cells was assessed by immunofluorescence ($n=4$; scale bar: 25 μm). (E) Immunofluorescence was quantified by the fluorescence intensity. (F,G) Protein expression of CFTR in 16HBE14o- cells treated with Y27632 was detected by western blot analysis ($n=3$). The experiments were performed three times, and data are presented as the mean ± SD. HDM: 10 μg protein in 50 μL saline; CN03: 1 μM, pFU-GW-007 was used as control. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

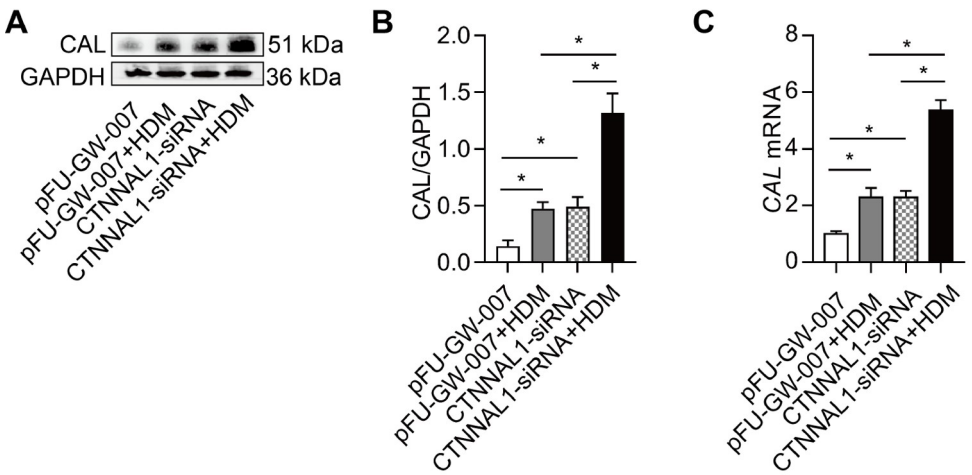

Figure 8. CTNNAL1 deficiency increased the expression of CAL in HBE cells (A,B) Protein expression of CAL in 16HBE14o- cells was detected by western blot analysis ($n=5$). (C) mRNA expression of CAL in 16HBE14o- cells was detected by RT-qPCR ($n=5$). The experiments were performed three times, and data are presented as the mean ± SD, HDM: 10 μg protein in 50 μL saline, pFU-GW-007 was used as control. *$P<0.05$.

bicarbonate [33]. Mucus is highly dependent on the presence of bicarbonate for normal function, and reduced anion concentrations might cause derangements of mucus tethering and detachment, increasing the viscosity of airway mucus [34]. As a consequence, dysfunction of CFTR results in hydration, mucociliary clearance, mucus tethering and function, and impaired innate immunity. Based on our previous studies, CTNNAL1 was downregulated in asthmatic patients and participated in the wound repair and proliferation of HBE cells [8,11]. We propose that it plays an important role in maintaining airway epithelial cell homeostasis. CTNNAL1 deficiency enhances lung inflammation and mucus hypersecretion [11]. Upon HDM stimulation, we observed that CTNNAL1 deficiency significantly increased the inflammatory response and mucus production. In this study, we found that CFTR

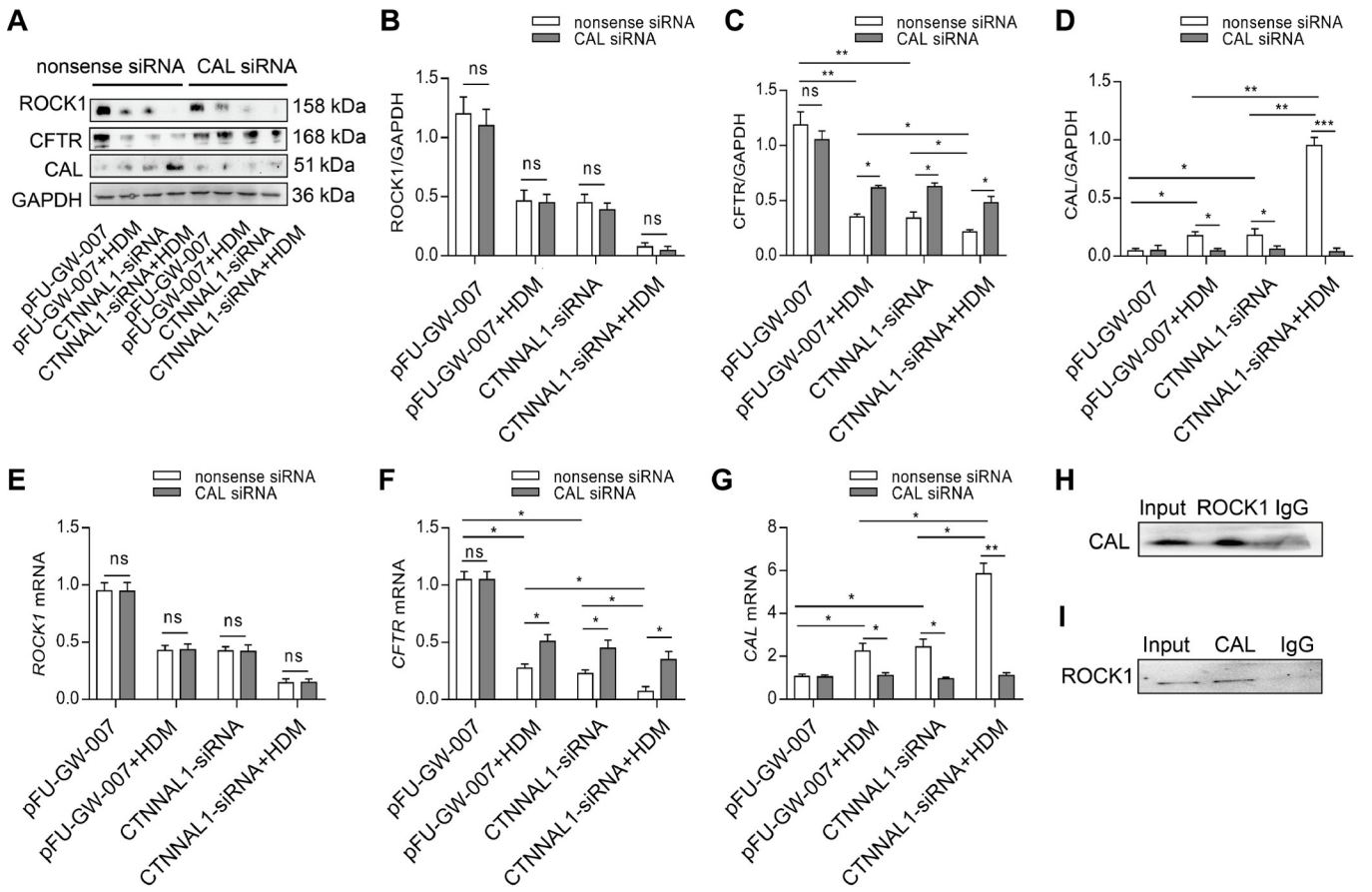

Figure 9. CAL inhibition upregulated CFTR expression in response to CTNNAL1 (A–D) Protein expression levels of ROCK1, CFTR and CAL in 16HBE14o- cells were examined by western blot analysis ($n=3$). (E–G) mRNA expression levels of ROCK1, CFTR and CAL were detected by RT-qPCR ($n=3$). (H,I) The interaction between CAL and ROCK1 was assessed by immunoprecipitation in airway epithelial cells ($n=5$). The experiments were performed three times, and data are presented as the mean ± SD; HDM: 10 μg protein in 50 μL saline; pFU-GW-007 was used as control. *$P<0.05$, **$P<0.01$, ***$P<0.001$. ns: no significance.

expression was decreased in the lungs of AAV5-CTNNAL1-RNAi mice and significantly decreased in CTNNAL1-deficient mice upon HDM stimulation. Previous studies have shown that airway mucus hypersecretion in asthmatics is associated with CFTR mutation [35], which is consistent with our results.

It has been reported that CFTR ΔF508 heterozygosity may be overexpressed among people with asthma and seems to be associated with decreased pulmonary function in people with airway obstruction who also have asthma [36]. However, some studies failed to find a higher incidence of asthma in CF carriers with the F508del mutation, which were carried out between 1998 and 2008 [37,38]. In 2012, Goodwin *et al.* [39] reported 4 cases of asthma and airway mucus hypersecretion, a neutrophilic inflammatory phenotype accompanied by bronchiectasis, pansinusitis, respiratory infections and mutations and/or polymorphisms in the CFTR gene, positing the interesting possibility of an association between mutations and a characteristic phenotype. Crespo-Lessmann *et al.* [40] demonstrated that patients with asthma and with mucus hypersecretion may have a different phenotype and disease mechanism produced by an intronic polymorphism in the CFTR gene.

CTNNAL1 was identified as a part of the Rho signaling pathway, serving as a scaffold protein for Lbc, and it enhances RhoA activity. ROCK is a downstream effector of RhoA [13]. There are two different ROCK forms, ROCK1 and ROCK2. They have the highest amino acid homology between their kinase domain (92%) and are most divergent within their coiled-coil domains with 55% homology [41]. In our previous study, we demonstrated that the expression of ROCK1 decreased while ROCK2 increased when *CTNNAL1* was silenced. Inhibition of ROCK2 decreased MUC5AC production and cytokine secretion after *CTNNAL1* was silenced [11]. The present data clearly showed that inhibition of ROCK1 decreased CFTR expression whether *CTNNAL1* was silenced or overexpressed. Additionally, we confirmed that activation of RhoA increased CFTR expression when *CTNNAL1* was silenced. Furthermore, Y27632 treatment decreased the expression level of CFTR in *CTNNAL1*-silenced HBE cells upon CN03 stimulation. These results demonstrated the involvement of ROCK1 in the CTNNAL1 deficiency-mediated decrease in CFTR expression.

A number of PDZ proteins have been identified to directly or indirectly bind with the C-terminus of CFTR [42]. One of these is CAL, whose overexpression in heterologous cells directs the lysosomal degradation of CFTR in a dose-dependent manner and reduces the amount of CFTR at the cell surface [22]. Furthermore, it has been confirmed that the interaction between CAL and CFTR is direct [43]. In this study, we noted that both CTNNAL1 deficiency

and HDM stimulation could increase the expression of CAL in HBE cells. CAL expression was significantly increased in *CTNNAL1*-silenced HBE cells upon HDM stimulation. Rho family member TC10 directs the trafficking of CFTR from the juxtanuclear region to the secretory pathway toward the plasma membrane, away from CAL-mediated degradation of CFTR in the lysosome [23]. Cdc42, also a member of the Rho family, is involved in the biosynthesis of CFTR. It stabilizes CFTR functions on the cell membrane by anchoring actin, promotes the endocytosis of CFTR and mediates its degradation in lysosomes [44]. Our results proved that ROCK1, a Rho downstream target, and CAL directly interact in HBE cells. Inhibition of CAL led to an increase in CFTR expression, but ROCK1 expression remained unchanged. These results indicated that CTNNAL1 regulates CFTR expression through the ROCK1-CAL signaling pathway.

In summary, we demonstrated that CTNNAL1 deficiency decreases the expression of CFTR by activating the ROCK1-CAL signaling pathway in both lung tissues and HBE cells.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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