Motile cilia are numerously present moving structures that are crucial for flow generation in the airway epithelium, brain ventricle, oviducts, and middle ear. Defects in formation, maintenance, and function of motile cilia can result in ciliopathies that usually manifest as multisystem problems (1, 2). Ciliary length adjustment is an important parameter for control of cilia-generated fluid flow; however, the mechanisms that optimize this process remain less defined. It is argued that cilia-generated fluid flow, and ciliopathies in Kif19a−/− mice.

Cilia are not merely microtubular structures but form highly specialized signaling compartments enriched with cellular messengers including cAMP and Ca2+. Adenylate cyclases 5 and 6 (AC5/6) are the major suppliers of cAMP in nonmotile cilia (7, 8). Primary ciliary length is known to be sensitive to intracellular CAMP-dependent upon AC5/6 activity (9). In the context of motile cilia, the role of CAMP is known to be as an important regulator of ciliary beating and organization of compartmental signaling (10, 11). Whether ACs and CAMP regulate motile cilia growth and development has not been explored before. Also, the dynamic and reversible nature of motile cilia length remain unrecognized. More recently, the role of autophagy has been redefined to selectively clear primary cilia-associated proteins in autophagosomes fused with lysosomes that control ciliogenesis and ciliary growth (12, 13). This prompted our interest in investigating whether Kif19a is an autophagy substrate that may become relevant to motile cilia growth. Autophagy can become triggered by activation of AMPK, a heterotrimeric complex of a catalytic (α) and regulatory (β and γ) subunits, because it stimulates mechanisms to conserve energy and resources (14, 15). Active AMPK (Thr172−AMPK) stimulates autophagy indirectly via negative regulation of mTORC1 kinase complex and can directly activate it through binding, phosphorylating, and activating autophagy-initiating kinase Ulk1 (16). Protein kinase A also negatively regulates autophagy by impeding AMPK–Thr172 activation through phosphorylation at Ser172 and hampering ULK1 activation (17). In this study, we identified that AC6 activity inhibits AMPK activity and controls Kif19a protein levels via regulation of autophagy to alter motile cilia length and function, which can be mimicked by pharmacologically targeting of the AMPK pathway. Our study identified an important signaling pathway that can be directly targeted to alter motile cilia length of potential relevance to cilia-associated disorders.

Results

AC6 regulated ciliary length in mammal

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and confirmed reduced AC protein expression using AC5/6 antibody in isolated Adcy6\textsuperscript{-/-} Shh Cre/\textsuperscript{+} (Adcy6\textsuperscript{-/-}) mice tracheal epithelial cells (Fig. 1B). Considering a known role of cAMP in mucociliary clearance, we tested cilia-generated flow in Adcy6\textsuperscript{-/-} mice and observed a 2.4-fold lower particle movement in Adcy6\textsuperscript{-/-} compared with Adcy6\textsuperscript{+/-} mice trachea (Fig. 1, C and D, and Movie S1). Based on this observation, we considered the possibility of cilia-associated defects in Adcy6\textsuperscript{-/-} mice. Immunostaining data revealed apical enrichment of AC6 in the large airways (trachea) and localization not along the cilia but rather with the ciliary basal body marker γ-tubulin (Fig. 1, E and F). Based on our experimental determination, we hypothesized that defective ciliary movement in Adcy6\textsuperscript{-/-} tracheal epithelial cells could be in part due to longer cilia compared with the control mice (30% longer; 3.9 ± 0.7 in Adcy6\textsuperscript{+/-} versus 5.1 ± 0.7 in Adcy6\textsuperscript{-/-}) (Fig. 1, G and H). We were able to recapitulate these findings using PHBECs. We measured the length of ciliary tuft and isolated cilia and observed an ~50% increase in the cilia length upon lentiviral knockdown of AC6 expression using two separate shRNAs (Fig. 1, J and I). Based on these findings, we can conclude a prominent mucociliary clearance defect and elongated cilia upon loss of AC6 in the airway epithelium.

**AC6 knockout mice cilia are deficient in Kif19a**

Niwa et al. (6) identified Kif19a as a ciliary tip protein that acts as microtubule depolymerizing kinesin at the distal end of the cilia to regulate length. We observed that Kif19a was enriched at the ciliary tips and colocalized with ciliary tip marker EB1 but also expressed along the length of the cillum (Fig. 2A). We observed that lentiviral knockdown of Kif19a significantly increased ciliary length by >2-fold in PHBECs and mouse tracheal epithelial cells (MTECs) grown on the Transwells (Fig. 2, B–D). Therefore, we were able to confirm that Kif19a is a direct negative regulator of ciliary length as demonstrated previously (6). Based on our earlier observation of longer cilia in Adcy6\textsuperscript{-/-} tracheal cells, we proceeded to investigate whether Kif19a is a possible target of AC6 in controlling ciliary length. Interestingly, Kif19a protein levels were dramatically reduced in Adcy6\textsuperscript{-/-} trachea compared with the control, which was not at the level of transcripts (Fig. 2, E and F and Fig. S1A). Consistent with the observations above, transient overexpression of AC6 increased His-S-Kif19a (isoform 2, ~70 kDa) levels by 2-fold that did not concur with the possibility of reduced proteosomal degradation (Fig. 2, G and H, and Fig. S1B). Until this point, we can conclude that down-regulation of AC6 expression phenocopies loss of Kif19a.

**Kif19a is an autophagy substrate**

We next asked how AC6 affects Kif19a protein levels. Given the recently established role of autophagy in ciliogenesis (10, 11) and the interconnected AMPK–autophagy pathways, we proceeded to test whether the components of autophagy and AMPK pathway are altered in Adcy6\textsuperscript{-/-} epithelial cells. Indeed, up-regulated AMPK signaling, for example, increased P-AMPK-α–Thr\textsuperscript{172} and down-regulation of P-mTOR were observed in Adcy6\textsuperscript{-/-} isolated MTECs relative to the control (Fig. 3, A and B). Also, autophagy was up-regulated in Adcy6\textsuperscript{-/-} tracheal cells as indicated by increased lipidation of LC3 (LC3-II), an autophagy marker (Fig. 3, A and B). Next, we tested the possibility that AMPK-mediated autophagy might regulate Kif19a expression in Adcy6\textsuperscript{-/-} epithelial cells. First, we identified an interaction between Kif19a and the endogenous and overexpressed P-AMPK-α subunit (Fig. 3, C and D, and Fig. S2). Second, coexpression of recombinant AC6 compromised an interaction of Kif19a with AMPK-α (Fig. 3, C and D). Also, lowered P-AMPK-α and in the presence of AC6 overexpression suggested that AC6 impedes AMPK activation as corroborated by our findings in Fig. 3 (A and B). Importantly, we concluded that Kif19a preferentially interacts with the P-AMPK or active AMPK as Kif19a interaction with total AMPK followed an interaction pattern of P-AMPK (Fig. 3, C and D). Next, we investigated how modulation of AMPK activity may affect Kif19a protein levels. Treatment of cells with the inhibitor of AMPK activity, compound C enhanced while AMPK activator-AICAR decreased, Kif19a protein expression without altering the total levels of acetyl-CoA carboxylase, a direct AMPK target (Fig. 3, E and F). To investigate whether Kif19a is cleared in the autophagosomes because of an increased autophagy in AC6 KO tracheal cells, we tested Kif19a protein levels in cells treated with pharmacological activators (Torin1) and inhibitors (chloroquine (CQ)) of autophagy and in cells deficient for autophagy (generated upon knockdown of ATG5 expression) (Fig. 3, E–I). The incubation of cells with Torin1 decreased Kif19a protein expression in a dose-dependent manner, and Kif19a protein expression was increased in cells treated with both CQ and ATG5 sh (Fig. 3, E–I). To test whether AMPK drives Kif19a recruitment into autophagosomes, we performed in vitro colocalization study between Kif19a and LC3 in MTECs. An extensive accumulation of Kif19a was observed in LC3-positive autophagosomes in Adcy6\textsuperscript{-/-} MTECs that was marginal in Adcy6\textsuperscript{+/-} MTECs (Fig. 3, J and K). Inhibition of AMPK activity stalled Kif19a localization into autophagosomes in Adcy6\textsuperscript{-/-} and Adcy6\textsuperscript{+/-} MTECs, whereas AMPK activation enhanced this process in Adcy6\textsuperscript{+/-} MTECs (Fig. 3, J and K). In summary, AC6 down-regulates AMPK activation and prevents P-AMPK-Kif19a protein interaction, and AMPK activation drives Kif19a recruitment into autophagosomes for clearance.

**Loss of AMPK restores ciliary length in AC6 knockout mice**

Next, we investigated how the above described mechanism might translate into ciliary length regulation. Ciliary length negatively correlated with the endogenous Kif19a expression (determined using immunofluorescence) in MTEC cilia (Fig. 4A). Inhibition of AMPK reversed longer cilia in Adcy6\textsuperscript{-/-} MTECs that correlated with increased Kif19a expression (Fig. 4B). Treatment of tracheal cells with AICAR significantly increased the length of Adcy6\textsuperscript{-/-} cilia correlating with decreased Kif19a expression (Fig. 4B). This effect of AICAR was blunted in Adcy6\textsuperscript{-/-} cilia, suggesting that the activation of AMPK pathway in Adcy6\textsuperscript{-/-} tracheal cells may already be saturated, and also, no apparent change in Kif19a expression was observed under these conditions (Fig. 4B). These findings suggest that loss of Kif19a expression coincides with abnormal elongation...
EDITORS' PICK: Ciliary length regulation in mammals

A

RNAseq

PHBEC

MTEC

Relative AC expression

Adcy

1 2 3 4 5 6 7 8 9 10

B

KDa

t/t

t/t

Δ/Δ

Δ/Δ

AC5/6

AC5/6

MTEC

GAPDH

C

Adcy6'

Adcy6
d

Flow direction

Red: Bead tracks

E

Mouse trachea

AC5/6 DNA

G

Trachea

f/f

Δ/Δ

Trachea

f/f

Δ/Δ

Ac tub

H

Cilia length (µm)

****p < 0.0001

I

Isolated cilia from PHBEC

Ac tub + γ-tub

Ph sh

AC6 sh1

AC6 sh2

J

Cilia length (µm)

****p < 0.0001

****p < 0.0001

****p < 0.0001

****p < 0.0001

****p < 0.0001
of Adcy6ΔΔ cilia. Also, the AC6 and AMPK pathways cooperatively act to regulate motile cilia length. The abnormally long cilia measured in Adcy6ΔΔ ex vivo trachea correlated with poor cilia-generated flow (Fig. 4C). Treatment of Adcy6ΔΔ tracheal samples with Comp. C (4 h) improved cilia-elicited bead velocity that correlated with shorter cilia length. Treatment of Adcy6ΔΔ tracheal samples with AICAR (4 h) impaired cilia-generated flow correlating with longer cilia (Fig. 4C). Hence, there is a clear negative correlation between longer cilia height and impaired cilia-generated flow. Overall, these findings imply that the abnormal cilia-generated flow in Adcy6ΔΔ Airways can be at least explained by longer than normal cilia in these mice.

**Discussion**

Our present a previously unidentified mechanism of dynamic regulation of motile cilia length (Fig. 4D). Previous study showed that there is a dose dependence of Kif19a to cilia length (6). Because Kif19a is a plus-end-directed motor, plus-end being the growing distal end of cilia, Niwa et al. (6) suggested that this means a more efficient way to regulate cilia length compared with both plus- and minus-end-directed microtubule rearrangement (6). Based on the marked ciliary phenotype in Kif19a+/− mice as reported earlier, it is very unlikely that there is a redundancy to the role of Kif19a, and it seems to be conserved. This makes Kif19a a key determinant of the optimal length of motile cilia in mammals. It is suggested that Kif19a depolymerizes microtubules in a length-dependent manner that is important for the dynamic aspect of ciliary growth (18).

Autophagosomes carry highly selective protein cargos for degradation that becomes crucial to the homeostatic balance of the cell. Several of these cargos are recruited based on their interaction with the specific components of the autophagic machinery, and in this manner, autophagy can be a selective pathway. LC3-LC2, a specific autophagy marker, serves as the membrane receptor to select and recruit cargoes for lysosomal degradation. Autophagy has recently been shown to function in the growth of primary cilia (13). LC3 has been demonstrated to interact with antibodies to core microtubules of mammalian cilia in ciliated cells and made into effect by up-regulated AMPK-autophagy pathway.

Our findings identified key molecular factors that cooperatively regulate Kif19a levels to mediate ciliary length control in mammals. This study provides an important piece of evidence to support inherent plasticity in motile cilia that may be crucial during key developmental events and in adult mammalian function.

**Experimental procedures**

**Mice**

Adcy6 ΔΔ mice were obtained from The Jackson Laboratory following cryorecovery and were interbred to obtain Adcy6 ΔΔ mice. Adcy6 ΔΔ mice were then crossed with Shh GFP cre mice (B6.Cg-ShhΔ Svg1EGFP/creRt j, The Jackson Laboratory); following a series of intercrosses, Adcy6 ΔΔ mice (Adcy6 ΔΔ ShhΔ Svg1EGFP/creRt ΔΔ mice) were obtained. The male mice were used as Shh GFP cre donors. As a control to Adcy6 ΔΔ mice, littermate Adcy6 ΔΔ mice were used. Typically, 6–12-week-old male mice were used for the study. All the mice were maintained in a barrier facility at Cincinnati Children’s Hospital Medical Center and were fed normal chow. All procedures were performed in compliance with institutional guidelines and were approved by the institutional animal care committee.
EDITORS' PICK: Ciliary length regulation in mammals

A Isolated cilia

B Isolated cilia

C Isolated cilia + Cilia tuft

D MTEC

E Mouse trachea

F

G + + + + FLAG-AC6

H Hist-S-Kif19a

**p < 0.01

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by Cincinnati Children’s Hospital Medical Center’s Institutional Animal Care and Use Committee.

**Chemicals and antibodies**

Antibodies in this study included AC5/6 (Santa Cruz), Kif19a (Proteintech), acetyl-tubulin (Sigma), γ-tubulin (Sigma), LC3 (Sigma), β-actin (Sigma), AMPK and mTOR sampler kit (Cell Signaling Technologies), GAPDH (Cell Signaling Technologies), S-protein HRP (EMD Milliipore), IFT88 (EMD Millipore), and ubiquitin (CST). AMPK activator 5-aminimidazole-4-carboxamide ribonucleotide (AICAR), inhibitor compound C (Dorso-morphin), and Torin1 were purchased from Selleck Chemicals (Houston, TX, USA). Autophagy inhibitor chloroquine was obtained from MP Biomedicals (Santa Ana, CA, USA).

**Cell transfection**

HEK 293 and 16 HBE14o- cells were obtained from ATCC. HEK 293 and 16 HBE14o- cells were cultured in DMEM/F-12 and minimal essential medium, respectively, containing 10% FBS and 1% penicillin–streptomycin. Kif19a cDNA isoform 2 was obtained from GE Dharmacon (Lafayette, CO, USA) with a molecular mass coming close to 70 kDa (the molecular mass of the native form of Kif19a is 111 kDa) and inserted into pTriEx vector backbone. pTriEx Kif19a cDNA was transfected by using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. Transfected cells were studied after 48 h. Primary human bronchial epithelial cells were obtained as differentiated on Transwells from two sources: the Cystic Fibrosis Center of the Cincinnati Children’s Hospital Medical Center and Charles River Laboratories (Wilmington, MA, USA).

Ready-to-transduce GPIZ lentiviral shRNA particles (mouse and human scrambled AC6 and Kif19a shRNA) were obtained from GE Dharmacon. Primary human bronchial epithelial cells were treated with the 5 \times 10^5 transduction units viral particles for 48 h on the filters (added to the basolateral side) followed by 48 h of recovery and then again treated with 2.5 \times 10^5 transduction units viral particles for 48 h before the experiment. At least two shRNAs were used for AC6 and Kif19a in human cells. For murine cells AC6 and Kif19a shRNAs were used singly or in a combination of two. A 50–60% knockdown efficiency was obtained using these shRNAs.

PHBECs were generated from three donors from the two sources combined. Transduction controls comprised of either nonsilencing lentiviral particles or GAPDH shRNA.

**Mouse tracheal epithelial cell culture**

Protocol for isolation of tracheal cells was designed with slight modifications from a previously established method (21). Briefly, the trachea were collected in ice-cold Ham’s F-12/pen–strept/1× fungizone, and connective, muscular, and vascular tissues were removed manually using forceps. The tracheal tube was opened longitudinally and washed twice in Ham’s F-12. Tracheal tissue was incubated in Ham’s F-12 with 0.15% Pronase at 4°C and kept upright. Glass-bottomed dishes (Maktek) or plastic culture plates were coated with 50 μg/ml type I rat tail collagen in 0.02 N acetic acid for 18 h at room temperature. The next day, the tissue was transferred into 10 ml of Falcon containing 10 ml of DMEM/F-12 medium with 10% FBS, 1% pen–strept and pipetted up and down several times. Supernatant was collected and spun at 1200 rpm for 10 min. The pellet was resuspended in 200 μl of Ham’s F-12/pen–strept containing crude pancreatic DNase I 500 μg/ml, 0.1% BSA and incubated on ice for 5 min. Next, 10 ml of DMEM/F-12 medium is added and spun at 1200 rpm for 10 min. The pellet is resuspended in 3 ml of DMEM medium and kept for 3–4 h in 5% CO₂ at 37°C to remove fibroblasts. Nonadherent cells were collected and centrifuged at 1200 rpm for 5 min at 4°C. The cell pellet was resuspended in mouse tracheal epithelial cell culture medium ((Ham’s F-12, 1% penicillin–streptomycin, 5% FBS, 1 × fungizone (Stock 1000 ×), insulin (10 μg/ml), transferrin (5 μg/ml), mEGF (25 ng/ml), bovine pituitary extract (30 μg/ml), chola toxin (0.1 μg/ml), and retinoic acid (0.5 nm)) for 1 h. Then, the cells were counted and plated in 2D culture or on the costar Transwells.

**Cilia-generated flow measurement**

Cilia-generated flow measurement in the whole trachea was performed as described previously (22). Briefly, whole trachea were excised from mice and bathed in Leibovitz’s L-15 medium containing 10% FBS. Thin tracheal rings were cut under a M165FC stereomicroscope (Leica Biosystems, Wetlar, Germany) and mounted in 1-mm-spaced slice anchors (Warner Instruments, Hamden, CT, USA). The samples were placed under BX61WI fixed-stage motorized upright microscope (Olympus, Tokyo, Japan). FITC-conjugated beads (0.5 μm) were added to the samples. Bead flow was measured at 32 frames/s. Quantitation of bead velocity was performed as described earlier (22).

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**Figure 2.** AC6 KO mice airway cilia are deficient in ciliary kinesin Kif19a. A, isolated cilia from PHBECs immunostained for Kif19a (green) and acetyl tubulin (Ac tub) + γ-tubulin (γ-tub, red, left panel; scale bar, 5 μm) and EB1 (blue), Kif19a (gray), and acetyl tubulin (red, right panel; scale bar, 5 μm). B, left panel, isolated cilia from PHBECs transfected with GAPDH sh and Kif19a sh1/sh2 (scale bar, 2 μm). Middle panel, immunostained for ciliary tip marker EB1 (green) and acetyl tubulin (red). Right panel, DIC images of ciliary tuft from PHBECs transfected with GAPDH sh and Kif19a sh1/sh2 (scale bar, 5 μm). C, quantitation of cilia length PHBECs transfected with either GAPDH shRNA or Kif19a shRNA lentiviruses (n = 3 separate Transwells). Each dot represents an average of a single ciliary tuft with 5–10 cilia or an average of five isolated cilia. D, MTECs isolated from WT mice and treated with control shRNA or Kif19a shRNA lentiviral particles (n = 3 independent cell preparation). Each dot represents an average of five cilia. E, Western blotting data plotted using Kif9a antibody (top panel) demonstrate down-regulation of Kif19a protein expression in Adocy6^{Δ/Δ} compared with Adocy6^{+/+} trachea. Acetyl tubulin (bottom panel) was used as a loading marker. F, quantitation of Kif19a protein expression in Adocy6^{Δ/Δ} compared with Adocy6^{+/+} trachea from the Western blotting as represented in E (n = 4 mice/group). G, Western blotting probed using S-protein-HRP antibody demonstrates up-regulation of His-S-Kif19a protein expression in 16 HBE14o- cells with and without overexpression of FLAG-AC6. β-Actin (bottom panel) was used as a loading marker. H, quantitation of His-S-Kif19a expression in HEK 293 cells (n = 5 independent experiments) as represented in the Western blotting in G. Error bars represent S.E. Student’s t test was used for a pair-wise comparison with P < 0.05 considered significant. For multiple comparisons one-way ANOVA was used with P value adjusted using Bonferroni’s factor.
cilia-generated flow marked by bead velocity and ciliary length (determined in hematoxylin/eosin staining) in treatment of 16 HBE14o

Figure 3. AMPK pathway regulates expression of ciliary kinesin Kif19a.

Cell lysate preparation and coimmunoprecipitation

His-S-Kif19a, S-protein–agarose was added to the whole cell lysate and mixed overnight at 4 °C. Proteins immobilized on beads were eluted using 5× sample buffer and subjected to SDS–PAGE and Western blotting. Whole lung cells and MTECs were lysed in radioimmune precipitation assay buffer

Figure 4. AMPK pathway regulates motile cilia length correlating with altered Kif19a expression. A, dot plot to show negative correlation determined using Pearson coefficient (r) between ciliary length (based on acetyl tubulin staining) and Kif19a expression (Kif19a-specific immunostaining) in a total of 159 ciliary tufts. B, dot plot to show negative correlation determined using Pearson coefficient (r) between ciliary length (based on acetyl tubulin staining) and Kif19a expression (Kif19a-specific immunostaining) in Adcy6f/f and Adcy6ΔΔ MTECs on Transwells treated with compound C (5 μM, 24 h) and AICAR (1 mM, 24 h) (n = 23–60 ciliary tufts). C, dot plot to show negative correlation determined using Pearson coefficient (r) between cilia-generated flow marked by bead velocity and ciliary length (determined in hematoxylin/eosin staining) in Adcy6f/f and Adcy6ΔΔ tracheal rings treated with compound C (10 μM, 4 h) and AICAR, (1 mM, 4 h) (n = 3–5 tracheal rings from 3 mice/group). D, model of dynamic regulation of motile cilia length in which AGC acts a checkpoint for ciliary growth via negative regulation of AMPK activity to prevent autophagic clearance of ciliary depolymerizing kinesin Kif19a. Error bars represent S.E.
(CST) containing protease inhibitors. Endogenous Kif19a was immunoprecipitated from whole-lung lysate using anti-Kif19a antibody cross-linked to protein A–agarose (0.5 μg of antibody per 10 μl of protein A–conjugated resin by using disuccinimidyl suberate cross-linker). Proteins immobilized on beads were eluted using a low-pH, glycine-based elution buffer containing 0.2% Triton X-100. The samples were incubated for 10 min at 37 °C and subjected to SDS–PAGE and Western blotting.

Immunoﬂuorescence

PHBECs and MTECs were fixed using 3.7% formaldehyde for 15 min at 25 °C. The cells were then washed and permeabilized using PBS with 0.3% Triton X-100 for 30 min at 25 °C. After a brief wash, the cells were blocked with 2% BSA–PBS–Tween-20 for 1 h at 25 °C. The cilia were stained with acetyl-tubulin antibody (1:1000). Alexa Fluor–conjugated antibodies were used for detection. The images were taken using a confocal microscope (Olympus FV1200). In the immunoﬂuorescence controls, no antibody was added to the samples.

Real-time PCR

RNA was extracted using Qiagen RNasy mini kit. cDNA was synthesized using SuperScript III reverse transcriptase (Thermo Scientific). Sybr green-based real-time PCR was performed using prevalidated primers for selected murine genes.

Statistics

At least three independent experiments were performed for quantitative measurement. Error bars represent S.E. Student’s t test was used for a pair-wise comparison with two-tailed analysis, and one-way ANOVA was applied for multiple comparisons. The P value was adjusted using Bonferroni’s correction. A P value <0.05 was considered signiﬁcant.

Data availability

All the data are included in the article.

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Conflict of interest—The authors declare that they have no conﬂicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AC, adenylate cyclase; KO, knockout; AMPK, AMP-activated kinase; PHBEC, primary human bronchial epithelial cell; MTEC, mouse tracheal epithelial cell; P-, phosphorylated; C, chloroquine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DMEM, Dulbecco’s modiﬁed Eagle’s medium; FBS, fetal bovine serum; pen–streptomycin; ANOVA, analysis of variance; DIF, differential interference contrast.

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