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Rab5c promotes RSV and ADV replication by autophagy in respiratory epithelial cells

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ABSTRACT

Respiratory system diseases caused by respiratory viruses are common and exert tremendous pressure on global healthcare system. In our previous studies, we found that Long non-coding RNA NRAV (Lnc NRAV) and its target molecule Rab5c plays a significant role in respiratory virus infection. However, the mechanism by which Rab5c affects virus replication remains unclear. Rab5c, a protein mainly localized on the cell membranes and in early endosomes and phagosomes, participates in endocytosis mediated by clathrin and regulates the fusion of early endosome, maturation of early phagosomes, and autophagy. Therefore, we inferred that Rab5c impacts virus replication, which might be related to endocytosis or autophagy. We selected RSV (respiratory syncytial virus) as a representative enveloped virus and ADV (Adenovirus) as a representative non-enveloped virus to explore the possible mechanism of RSV and ADV replication promoted by Rab5c in A549 cells and in Rab5c-overexpressing mice. Here, we confirmed that the activated Rab5c promotes RSV and ADV replication and the inactivated Rab5c inhibits their replication. However, Rab5c promoting RSV and ADV replication is not mediated by endocytosis rather by autophagy in respiratory epithelial cells. Our study showed that Rab5c upregulates LC3-II (microtubule-associated protein 1 light chain 3 beta) protein expression levels by interacting with Beclin1, a key autophagy molecule, which can induce autophagy and promote replication of ADV and RSV. This study enriches the understanding of the interaction between respiratory viruses and Rab5c, providing new insights for virus prevention and treatment.

1. Introduction

Respiratory viruses can cause respiratory diseases, including local respiratory lesions in mild cases and systemic tissue and organ damage in severe cases. The viruses, such as COVID-19, have brought enormous pressure to health service system in the world. Respiratory syncytial virus (RSV) is one of them. In our previous research, we focused on the immune regulation associated with RSV infection, and found that long non-coding RNA NRAV (Lnc NRAV) was significantly correlated with RSV infection (Li et al., 2020). Lnc NRAV played a molecular sponge role by adsorbing and binding to miR-509–3p, releasing the inhibition of miR-509–3p on Rab5c mRNA, and successfully releasing Rab5c mRNA for translation and expression to exert its function (Li et al., 2020; Ouyang et al., 2014). Rab5c is an important target molecule for Lnc NRAV to exert regulatory effects, but the mechanism by which Rab5c affects viral replication remains unclear.

Rab5c is a member of the Rab protein family, mainly localized on cell membranes and in early endosomes and phagosomes (Carney et al., 2006). Rab proteins, the largest subgroup of small GTPases in the Ras superfamily, have two different structural forms, namely the active state bound to GTP and the inactive state bound to GDP. Rab5 can participate in various functions such as endocytosis mediated by clathrin and regulate early endosome fusion, maturation of early phagosomes, and autophagy (Germann and Alam, 2020). Virus coming into the host cells through the cell membrane is a key step in virus replication. Respiratory viruses can be divided into enveloped viruses and non-enveloped

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viruses, which are dominated by membrane fusion and endocytosis respectively. After entering the cell, most viruses arrive at the early endosome and the late endosome successively, either releasing viral genomes to initiate replication or captured by autophagosomes to be degraded. However, many viruses have evolved a series of mechanisms that utilize autophagy to avoid degradation or even promote their own replication (Bento et al., 2016; Chiok et al., 2022). Accordingly, we speculated that Rab5c may affect virus replication through endocytosis or autophagy pathways. Based on these studies, we selected RSV as a representative enveloped virus and ADV as a representative non-enveloped virus to explore the effects of Rab5c on the replication of RSV and ADV *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals and treatment

Six-week-old female specific-pathogen-free (SPF) BALB/c mice were purchased from the Experimental Animal Center of Hebei Medical University. All mice were housed in temperature-controlled individual ventilated cages (IVC) with 12-h light/12-h dark cycles and were fed with standard chow and sterile tap water.

WT mice (n = 18) were divided into 3 groups (n = 6 per group), which were infected with PBS, AAV6-NC (1×10^{11} AAV virions, produced by GENECHEM) and AAV6-Rab5c (1×10^{11} AAV virions, produced by GENECHEM) by intranasal instillation. The overexpression of Rab5c were validated by western blot and IHF. Each group was randomly divided into 2 subgroups (3 mice/ per subgroup), and we infected the 2 subgroups of mice with RSV-GFP or ADV (1×10^{6} PFU per mouse) intranasally for 5 days. LC3II protein and GFP protein expressions were detected by Western blotting. The RSV N or ADV MLP mRNA relative expression levels were examined by qRT-PCR and viral titers were evaluated by plaque assay. Lung pathology was detected by H&E stain.

2.2. Cells and viruses

Human alveolar epithelial cells (A549) were cultured in RPMI 1640 medium (31,800–022; Gibco) supplemented with 10 % fetal bovine serum (FBS, 34894428S; Biological Industries), 10 mM HEPES (0511; Biosharp, Amresco), and 0.1 % penicillin-streptomycin solution (P1400; Solarbio) in a humidified 5 % CO_2 atmosphere at 37 °C. The pH of the cell culture media HEPES should be ajust for 7.2. RSV A strain Long from ATCC and GFP-RSV A strain, kindly provided by professor He Jinsheng (Beijing Jiaotong University), were amplified in Vero E6 cells. Viruses were harvested from the culture supernatant of Vero E6 cells at 72–96 h post-infection and stored at −80 °C until use. Adenovirus (ADV5; ADMax-GFP-NC; Gene Pharma) was amplified in 293A cells, harvested from the culture supernatant of 293A cells at 72–96 h post-infection, and stored at −80 °C until use.

2.3. Viral infection and virus titer assays

A549 cells were infected with RSV or ADV at a certain multiplicity of infection (MOI) or mock-infected (with medium alone) for 2 h in serum-free RPMI 1640. The supernatant was aspirated after adsorption, and cells were cultured with RPMI 1640 supplemented with 2 % FBS for the indicated times. Virus titers were determined using a plaque assay. Briefly, 10-fold serial dilutions of infected cell lysates or lung homogenates were incubated with A549 cells in 96-well plates for 2 h. Then, supernatants were removed and replaced with 1 % (wt/vol) methylcellulose semisolid culture medium at 37 °C in a CO_2 incubator for 4 to 7 days. The number of GFP signals in A549 cells were counted using microscope.

2.4. Cell transfection

Cells were seeded in six-well or twelve-well plates. Plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

2.5. Western blot analysis

Total cell lysates were prepared with a radio immune-precipitation assay (RIPA) in an ice bath for 30 min and supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF; BL507A; BioSharp) and phosphate inhibitors (P1260; Solarbio). The protein concentration was determined with a NanoDrop 2000c spectrophotometer (EW-83,061–12; Thermo Scientific). A total of 30 μg proteins was separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the bands were transferred to a polyvinylidene fluoride (PVDF) membrane (IPVH00010; Millipore), which was blocked with 5 % nonfat milk. After incubation with antibodies specific to Beclin1 (PD017; Medical & Biological Laboratories), human Rab5c (A7342; ABclonal), GFP(AB0005; Abways), LC3 (12741S; Cell Signaling Technology), β-actin (66,009–1-Ig; proteintech), and the blots were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (ASS1007; Abgent) or HRP-labeled goat anti-rabbit IgG (ASS1009; Abgent) and detected with Western Lightning plus-ECL reagent (NEL104001EA; PerkinElmer) using a Synoptics Syngene bio-imaging instrument (R114075; Synoptics). β-actin was used as a loading control for immunoblotting.

2.6. RNA extraction and reverse transcription quantitative PCR (qRT-PCR)

Total RNA from mouse tissues and cell lines was extracted with the TRIzol reagent (DP424; TIANGEN) according to the manufacturer's protocol. The RNA was reversely transcribed to cDNA using the PrimeScript RT reagent kit with genomic DNA (gDNA) Eraser (RR047A; TaKaRa). qRT-PCR was performed with cDNA templates and the PowerUp SYBR green master mix (A25741; Applied Biosystems), and analyzed using the ABI prism 7500 Sequence detection system (Applied Biosystems). β-actin was utilized as an internal standard control. The relative RNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The specific primers used are listed in Additional File 1: Table 1.

Table 1
Primers used in this study.

Primer name	Sequence
RSV F sense	5′-TAAGCAGCTCCGTTATCACATCTC-3′
RSV F antisense	5′-ATTGGATGCTGTACATTTAGTTTTGC-3′
RSV M sense	5′-ATGTGTAATGTGTCCTTGGATGA-3′
RSV M antisense	5′-TGATTTCACAGGGTGTGGTTACA-3′
RSV N sense	5′-AAGGGATTTTTGCAGGATTGTTT-3′
RSV N antisense	5′-CTCCCCACCGTAGCATTACTTG-3′
ADV MLP sense	5′-TTCCGCATCGCTGTCTG-3′
ADV MLP antisense	5′-CCGATCCAAGAGTACTGGAAAG-3′
ADV DBP sense	5′-ATCACCACCGTCAGTGAA-3′
ADV DBP antisense	5′-GTGTTATTGCTGGGCGA-3′
GFP sense	5′-ATGGTGAGCAAGGGCGAGGAG-3′
GFP antisense	5′-TCAAAGATCTACCATGTACAGCTCGT-3′
Rab5c sense	5′-AGGCTGCCATCGTGGTCTAT-3′
Rab5c antisense	5′-TTACCCGCGAGTGCAATGAC-3′
Mouse IL-1β sense	5′-ATGAAAGACGGCACACCCAC-3′
Mouse IL-1β antisense	5′-GCTTGTGCTCTGCTTGTGAG-3′
Mouse IL-6 sense	5′-AGACTTCCATCCAGTTGCCT-3′
Mouse IL-6 antisense	5′-TCTCCTCTCCGGACTTGTGAA-3′
Mouse TNF-α sense	5′-TGGCCTCCCTCTCATCAGTT-3′
Mouse TNF-α antisense	5′-TTGAGATCCATGCCGTTGGC-3′
Mouse β-actin sense	5′-CACTGCCGCATCCTCTTCCTCCC-3′
Mouse β-actin antisense	5′-CAATAGTGATGACCTGGCCGT-3′

2.7. Immunostaining and microscopy

A549 cells were seeded on coverslips and then transfected with mock or RSV or ADV infection (MOI = 3) for 24 h. The cells were fixed in 4 % paraformaldehyde, permeabilized in 0.5 % Triton X-100, and blocked in 10 % normal goat serum (SL038; Solarbio) for 30 min. The cells were incubated with primary antibodies anti-Rab5c (ab199530, 1:500) and anti-Beclin1 (PD017; Medical & Biological Laboratories, 1:50) overnight at 4 °C. Secondary antibodies included Alexa 594-labeled goat anti-rabbit (ab150080, 1:500) and Alexa 488-labeled goat anti-mouse (ab150113, 1:500). Coverslips were mounted on the glass slides with DAPI Flouromount-G (0100–20; SouthernBiotech) for confocal imaging (Leica TCS SP5).

2.8. CRISPR/Cas9-mediated gene knockdown

A549 cells stably expressing specific sgRNAs against Beclin1 were established by being transduced with lentiviral particles expressing specific sgRNA targeting Beclin1. Lentiviral particles were packaged by transfecting HEK 293T cells with YKO-sgBeclin1 Lentivectors (Ubigene Biosciences), psPAX2, and pMD2.G at a ratio of 4:3:1, respectively. Viral supernatants were collected at 48 h post-transfection. After infection with lentiviral particles or transfection, A549 cells were treated with hygromycin (200 μg/ml, BIOFROXX) or puromycin (3 μg/ml, Biosharp) for selection. Surviving cells were used as knockdown cells, and the knockdown efficiency was determined by western blotting. The sgBeclin1 sequence was as follows: gRNA:CCTGGATGGTGACACGGTCC AGG.

2.9. Lung histopathological examination

Mice lungs were perfused with PBS (phosphate buffer saline) and immediately preserved in 4 % paraformaldehyde overnight, dehydrated through a graded alcohol series, embedded in paraffin, cut into 5-micrometer-thick sections, and stained with hematoxylin and eosin (H&E). The slides were examined by light microscopy.

2.10. Coimmunoprecipitation (co-IP)

A549 cells transfected with the appropriate plasmids were harvested and lysed with IP-lysis buffer (P1045, Beyotime) with 1 mM PMSF and phosphatase inhibitors for 30 min at 4℃. Cell lysates were centrifuged at 13,400 × *g* for 20 min at 4 °C. The supernatant was collected, and protein concentrations were determined with a NanoDrop 2000 spectrophotometer. The protein extract was incubated with Anti-Flag affinity gel (P2271, Beyotime) overnight at 4℃. Then Anti-Flag affinity gel were collected and washed three times. Proteins binding on the beads were eluted with elution buffer (P9801, Beyotime) for 10 min, and finally analyzed with western blot.

2.11. Statistical analysis

Data from the three independent experiments were expressed as the mean ± standard deviation (SD). SPSS version 16.0 was used for statistical analysis. The significance between two groups was determined using Student's t-test. Statistical analysis for multiple groups were performed. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. The effect of Rab5c on the replication of RSV and ADV

To better understand the effect of Rab5c on respiratory virus infection, we transfected A549 cells with plasmids of activated Rab5c (Rab5c Q80L) or inactivated Rab5c (Rab5c S35N) and then infected GFP-tagged RSV and ADV. The results showed that Rab5c Q80L increased GFP protein expression in A549 cells infected GFP-RSV (Fig. 1A), total viral titers (Fig. 1B), the relative expression levels of RSV N, M, and F genes (Fig. 1C), and the number of green fluorescent cells (Fig. 1G). However, Rab5c S35N showed the opposite results (Fig 1D–G). Next, we evaluated the role of Rab5c Q80L and Rab5c S35N in GFP-ADV proliferation. Rab5c Q80L resulted in an increase in GFP protein expression (Fig. 1H), total viral titers (Fig. 1I), ADV MLP, DBP, and GFP gene expressions (Fig. 1J), and the lower GFP fluorescence intensity in GFP-ADV infected A549 cells (Fig. 1N). On the contrary, Rab5c S35N resulted in a decrease in these indicators (Fig1K-N). These results indicated that activated Rab5c could promote RSV and ADV replication, while inactivated form could inhibit their replication in A549 cells.

3.2. Endocytosis has no effect on Rab5c expression and Rab5c activity does not affect virus entry

Rab5c, as a protein mainly localized on the cell membranes, early endosomes, and phagosomes (Carney et al., 2006), participates in endocytosis mediated by clathrin and regulates the fusion of early endosome, maturation of early phagosomes and autophagy (Germann and Alam, 2020). It is well known that the virus can enter the cell via endocytosis, which is an early stage of the virus replication. However, it remains obscure whether endocytosis induced by virus infection can affect Rab5c expression. We detected the expression of Rab5c gene at the early stage of RSV and ADV infection and found that it reduced (Fig. 2A and B). Furthermore, we used endocytosis inhibitor Chlorpromazine (CPZ) to explore the effects of endocytosis on Rab5c expression. TF (Transferrin) enters into cells through receptor mediated endocytosis. We screened the appropriate working concentration of TF (Fig. 2D) through CCK8 experiments. Treatment the cells with CPZ at 25 μM/L, can effectively prevent TF from entering cells entering cells through endocytosis (Fig. 2E) with no cytotoxicity (Fig. 2C). In line with this, the fluorescence intensity of TF protein was attenuated (Fig. 2F). These results suggest that CPZ at the concentration of 25 μM/L could inhibit endocytosis without cytotoxicity. Subsequently, we used CPZ to detect that the effect of endocytosis on Rab5c expression in RSV or ADV infected A549 cells. The result showed that CPZ could not affect Rab5c expression in RSV or ADV infected A549 cells (Fig. 2G), which indicating that Rab5c expression was not regulated by endocytosis. Then we want to know whether Rab5c affects virus replication through endocytosis. To answer this question, we first identify the phase in which the virus enters the cell through endocytosis. The RSV N gene and ADV MLP gene expression were significantly increased within 2 h of virus infection, indicating that virus entered the cell within 2 h (Fig. 2H and I). Transfection with the plasmids of activated Rab5c or inactivated Rab5c don't affect RSV N gene and ADV MLP gene expression in virus-infected cells at 2 h (Fig. 2J and K), suggesting that Rab5c activity does not affect viral endocytosis. Totally, endocytosis has no effect on Rab5c expression, and *vice versa*.

3.3. Rab5c can affect virus replication through autophagy pathway

Our previous study demonstrated that RSV could induce complete autophagy (Li et al., 2018). And we verified this result again (Fig. S1A). However, it is still unclear whether ADV induces autophagy. In our study, ADV infection significantly increased the expression of autophagy marker protein (microtubule-associated protein 1 light chain 3 beta) LC3-II and decreased the expression of autophagy flux marker protein P62, suggesting that ADV infection could induce complete autophagy (Fig. S1B). As mentioned above, Rab5c promotes RSV and ADV replication without relying on the endocytosis, but it is still unclear whether Rab5c confer its pro-viral effects through autophagy. Firstly, we assessed whether Rab5c could induce autophagy. The result showed that Rab5c Q80L significantly increased LC3-II expression, while Rab5c S35N has no distinct effects (Fig. 3A), suggesting that Rab5c could induce

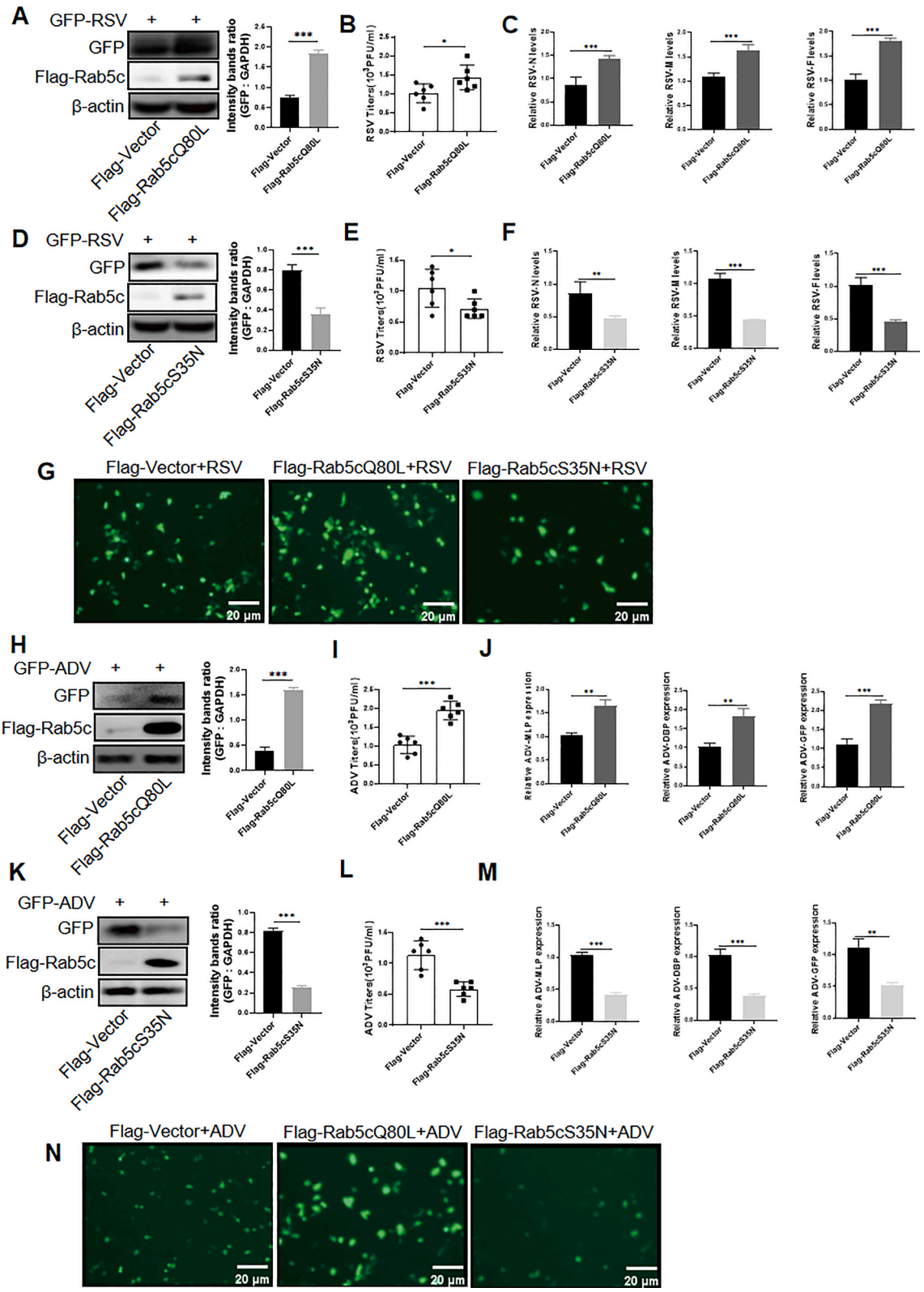

Fig. 1. The activated Rab5c promotes RSV and ADV replication and the inactivated Rab5c inhibits their replication.
(A–G) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with GFP-RSV for 24 h. The expressions of GFP protein were analyzed by Western blot (A and D). The total viral titers were detected by viral plaque assay (B and E). The relative expression levels of viral N, M, and F genes were detected by qRT-PCR (C and F). The GFP fluorescence of GFP-RSV was detected by fluorescence microscopy (G). Scale bar=20 μm.
(H—N) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with GFP-ADV for 24 h. The expressions of GFP protein were analyzed by Western blot (H and K). The total viral titers were detected by viral plaque assay (I and L). The relative expression levels of ADV genes were detected by qRT-PCR (J and M). The GFP fluorescence of GFP-ADV was detected by fluorescence microscopy (N). Scale bar= 20 μm.
(***P < 0.001, **P < 0.01, *P < 0.05*).

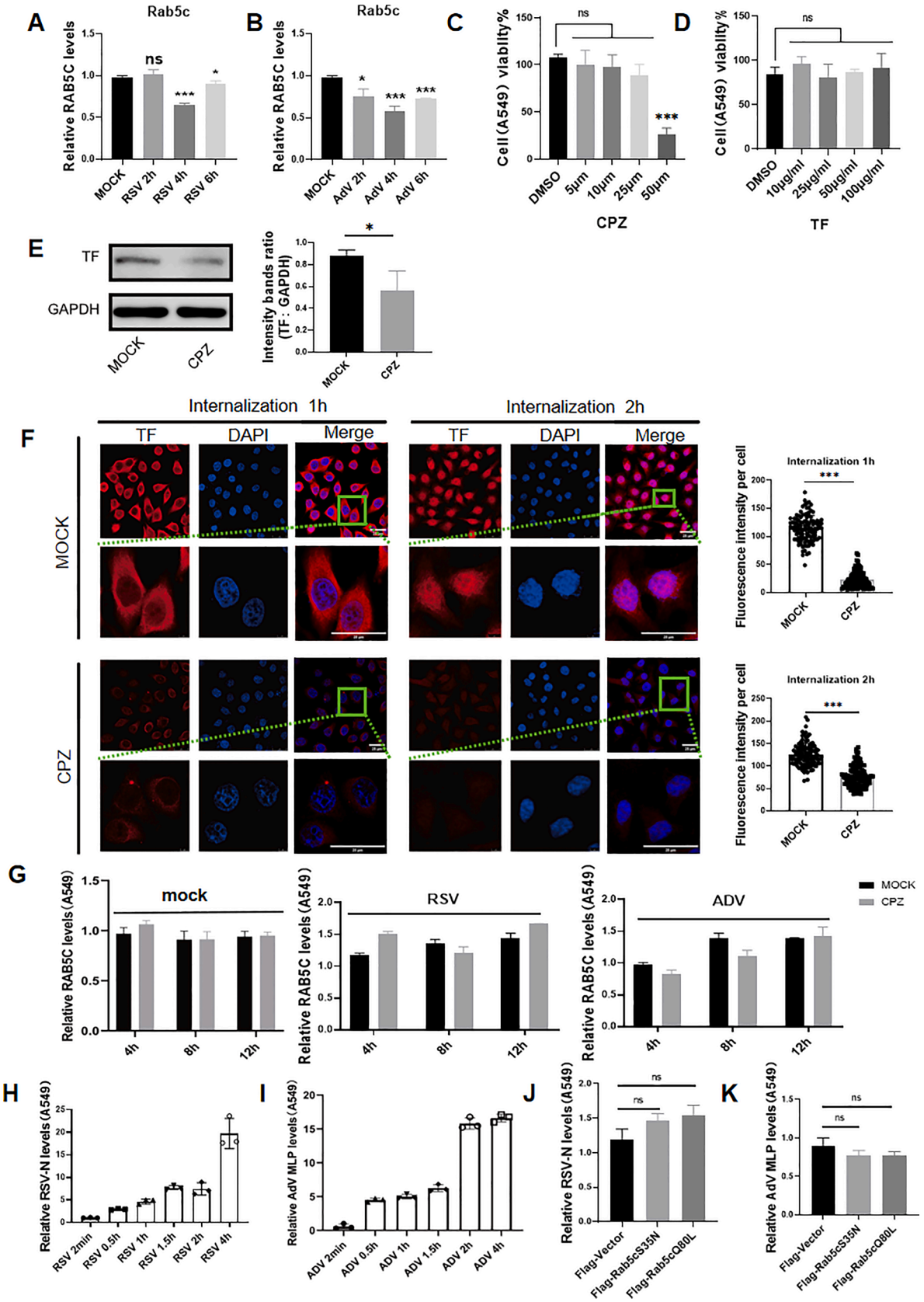

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Fig. 2. Endocytosis has no effect on Rab5c expression and Rab5c activity does not affect virus entry.
(A and D) A549 cells were infected with RSV(A) or ADV(D) for the indicated times. The relative expression level of Rab5c was detected by qRT-PCR.
(B and E) A549 cells were infected with RSV(B) or ADV(E) for the indicated times. The relative expression levels of RSV N(B) and ADV MLP(E) were detected by qRT-PCR.
(C and F) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with GFP-RSV for 24 h. The relative expression levels of RSV N(C) and ADV MLP(F) were detected by qRT-PCR.
(G and H) A549 cells were treated with different concentrations of CPZ(G) or TF(H), and cell viability was determined by CCK8 assay.
(I) A549 cells were treated with CPZ. The expression of TF proteins was analyzed by Western blot.
(J) A549 cells were mock-infected and infected with RSV or ADV for the indicated times in the absence or presence of CPZ. The relative expression level of Rab5c was detected by qRT-PCR.
A549 cells were treated with CPZ for 1 h and for 2 h. The fluorescence of TF was detected by fluorescence microscopy. Scale bar= 25 μm.
*(***P < 0.001, **P < 0.01, *P < 0.05)*.

autophagy. To further detect whether Rab5c promoted virus replication through autophagy, we pretreated the cells with autophagy inhibitors, bafilomycin A1 (Baf A1) and 3-Methyladenine (3-MA), and detected the effect of Rab5c on the virus replication. Treatment with Baf A1 at 20 μM/L and 3-MA at 50 μM/L with no cytotoxicity (Fig. 3B). The results showed that pro-viral effects of Rab5c Q80L and inhibitory effects of Rab5c S35N on virus replication, which were detected by the expression level of GFP protein and total virus titers, disappeared in GFP-RSV (Fig. 3C, D and E) and GFP-ADV (Fig. 3F, G and H) infected A549 cells treated with autophagy inhibitors. These results indicated that Rab5c promotes RSV and ADV replication by autophagy.

3.4. Rab5c can promote RSV and ADV replication by interacting with Beclin1

We have confirmed that Rab5c can promote viral replication by autophagy. However, the underlying mechanism of Rab5c inducing autophagy is not unclear. To further explore the mechanism, the cellular proteins combined with Rab5c were collected by immuno-precipitation with anti-Flag affinity gel from A549 cells which are transfected with a Rab5c Q80L plasmid and the cellular proteins were identified by immune-blotting. We found that Flag-tagged Rab5c could co-precipitate with Beclin1 in virus-infected A549 cells (Fig. 4A and B). In line with this, immunofluorescence showed that the co-localization between Rab5c and Beclin1 was also remarkably increased in virus-infected A549 cells (Fig. 4C). Besides, we explored whether Rab5c could interact with Beclin1 in A549 cells without virus infection. The immuno-precipitation assay showed that Flag-Rab5c Q80L could combine with Beclin1, while Rab5c S35N could not. Taken together, these results confirmed the interaction between Rab5c and Beclin1.In order to further explore whether Rab5c can promote RSV and ADV replication by interacting with Beclin1, we generated A549 cells with Beclin1 knocking down (A549- sgBeclin1 cells) with CRISPR/Cas9 (Fig. 5A). Firstly, we found that Rab5c could not induce autophagy in A549-sgBeclin1 cells (Fig. 5B). Secondly, the pro-viral effect of Rab5c on virus replication disappeared in A549-sgBeclin1 cells. Rab5c could not increase the expression of GFP protein of GFP-RSV or GFP-ADV (Fig. 5C and D), total viral titers (Fig. 5E and F) and the relative expression levels of RSV N and ADV MLP genes (Fig. 5G and H) in A549-sgBeclin1 cells. As mentioned above, Rab5c can promote RSV and ADV replication by interacting with Beclin1.

3.5. The relationship between Rab5c and respiratory virus replication in vivo

To confirm the role of Rab5c in autophagy and viral replication, we generated recombinant adenovirus expressing the Rab5c protein (AAV-Rab5c). We used 6-week-old female Balb/c mice which were infected with AAV-control or AAV- Rab5c by intranasal instillation for 30 days. The overexpression efficiency of Rab5c was validated by immune-blotting (Fig. S2A) and immunohistofluorescence (IHF) (Fig. S2B). Subsequently, AAV-control-infected and AAV-Rab5c-infected mice were infected with GFP-RSV or GFP-ADV intranasally for 5 days. Compared with that of AAV-control-infected mice at Day 5 post-infection (dpi), the levels of LC3-II and GFP protein expressions were increased in the lungs of the AAV-Rab5c-infected mice (Fig. 6A and B). Besides, the total viral titers of mice lungs (Fig. 6C and E), viral gene expression (Fig. 6D and F) and the mRNA expression levels of inflammatory cytokines IL-1β, IL-6, and TNF-α (Fig. 6G and H) were also increased in the lungs of the AAV-Rab5c-infected mice. In line with this, the hematoxylin and eosin (H&E) stain showed that lung pathology caused by RSV and ADV infection, which is characterized by lymphocyte infiltration and thickened alveolar septum, were significant aggravated in the AAV-Rab5c-infected mice (Fig. 6I). Those results showed that Rab5c could promote RSV and ADV replication *in vivo*.

4. Discussion

RSV (respiratory syncytial virus) and ADV (Adenovirus) are common viruses that cause respiratory tract infections, with RSV being one of the most common pathogens responsible for respiratory system diseases in children under the age of 5 worldwide (Li et al., 2022). Currently, there are no effective vaccines and specific drugs for treating RSV infection, which poses a significant burden on diagnosis and treatment globally (Mazur et al., 2023). This study is based on the previous experimental results that identified a high correlation between Lnc NRAV and RSV infection, as well as its target molecule Rab5c (Li et al., 2020).

Long non-coding RNAs (LncRNAs) are expressed in a conserved and tissue-specific manner (Palazzo and Koonin, 2020). In recent years, numerous studies have found that LncRNAs can participate in the regulation of various immune processes. In our previous study, we found a high correlation between Lnc NARV and respiratory virus infections. Although we observed decreased expression of Lnc NRAV in clinical specimens infected with various respiratory viruses such as RSV, IAV (Influenza A virus), IBV (Infectious Bronchitis Virus), and SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), some were not significantly affected, such as RV (Rabies Virus) and ADV (data not shown). In this study, we infected A549 cells with RSV and ADV and found that both viruses led to a decrease in Lnc NRAV and Rab5c expression.

Rab5c is a member of the Rab protein family, primarily localized on the cell membrane, in the early endosomes and phagosomes (Kumar et al., 2021). It is involved in the processes such as clathrin-mediated endocytosis, early endosome fusion, vesicle transport, and autophagy (Hermle et al., 2018; Redpath et al., 2019; Tu et al., 2022). Clathrin-mediated endocytosis is a common, non-specific mechanism used by many viruses to enter host cells. Based on these, we selected RSV as a representative enveloped virus and ADV as a representative non-enveloped virus in our study (Mah et al., 2023). We transfected A549 cells with pre-constructed plasmids of activated Rab5c (Rab5c Q80L) or inactivated Rab5c (Rab5c S35N) and then infected GFP-tagged RSV and ADV to investigate whether Rab5c affects viral replication. The experimental results showed that the activated form of Rab5c promoted the replication of RSV and ADV, while the inactivated form of Rab5c inhibited their replication. Our results suggested that Chlorpromazine (CPZ), an endocytosis inhibitor, could not affect Rab5c expression in

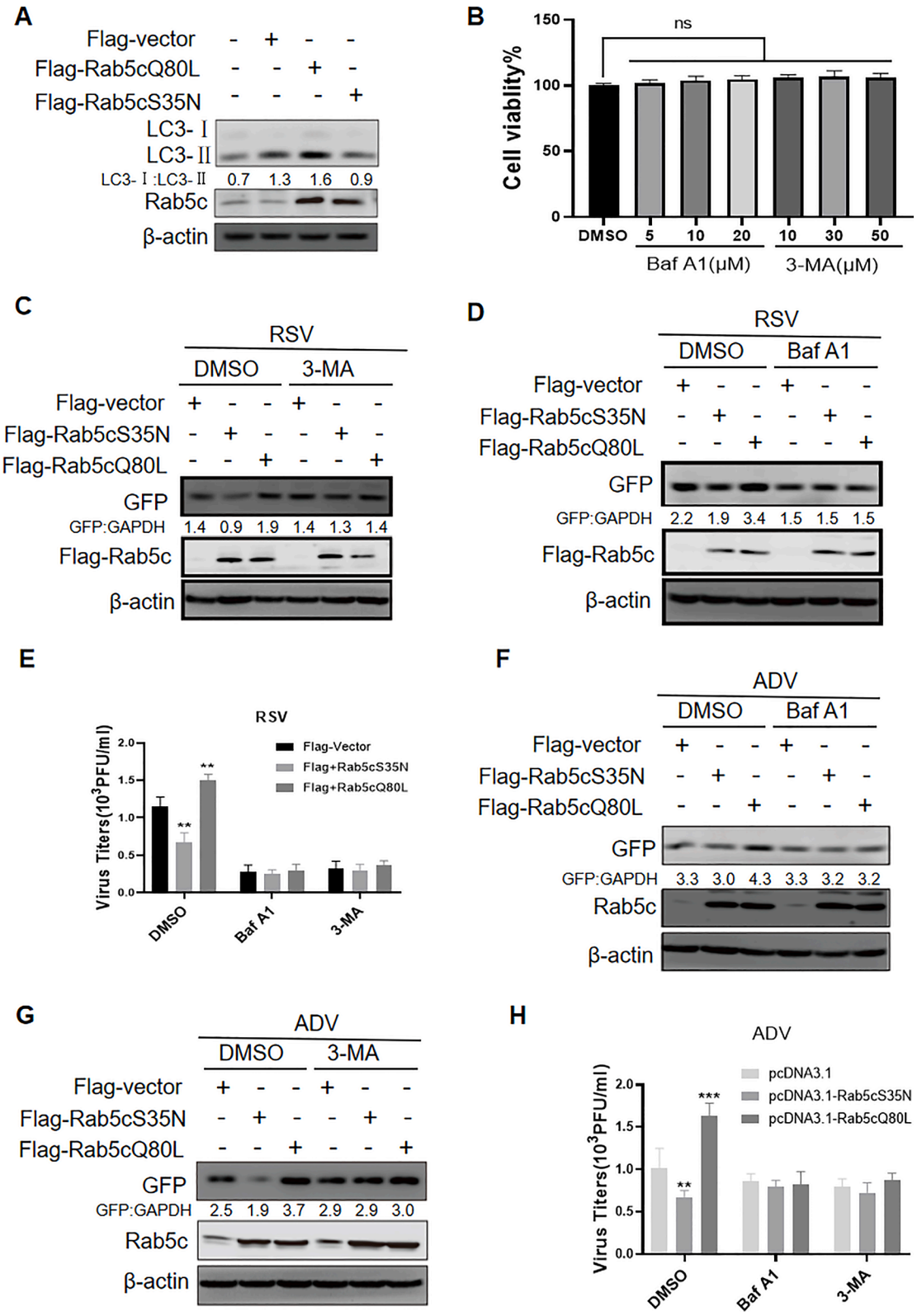

Fig. 3. Rab5c promotes virus replication through autophagy.
(A) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h. The expression of LC3-II was analyzed by Western blot.
(B–D) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with RSV for 24 h in the absence or presence of 3-MA or Baf A1. The expression of GFP protein was analyzed by Western blot (B and C). The total viral titers were detected by viral plaque assay (D).
(E–G) A549 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with ADV for 24 h in the absence or presence of 3-MA or Baf A1. The expression of GFP protein was analyzed by Western blot (E and F). The total viral titers were detected by viral plaque assay (G).
(***P < 0.001, **P < 0.01, *P < 0.05*).

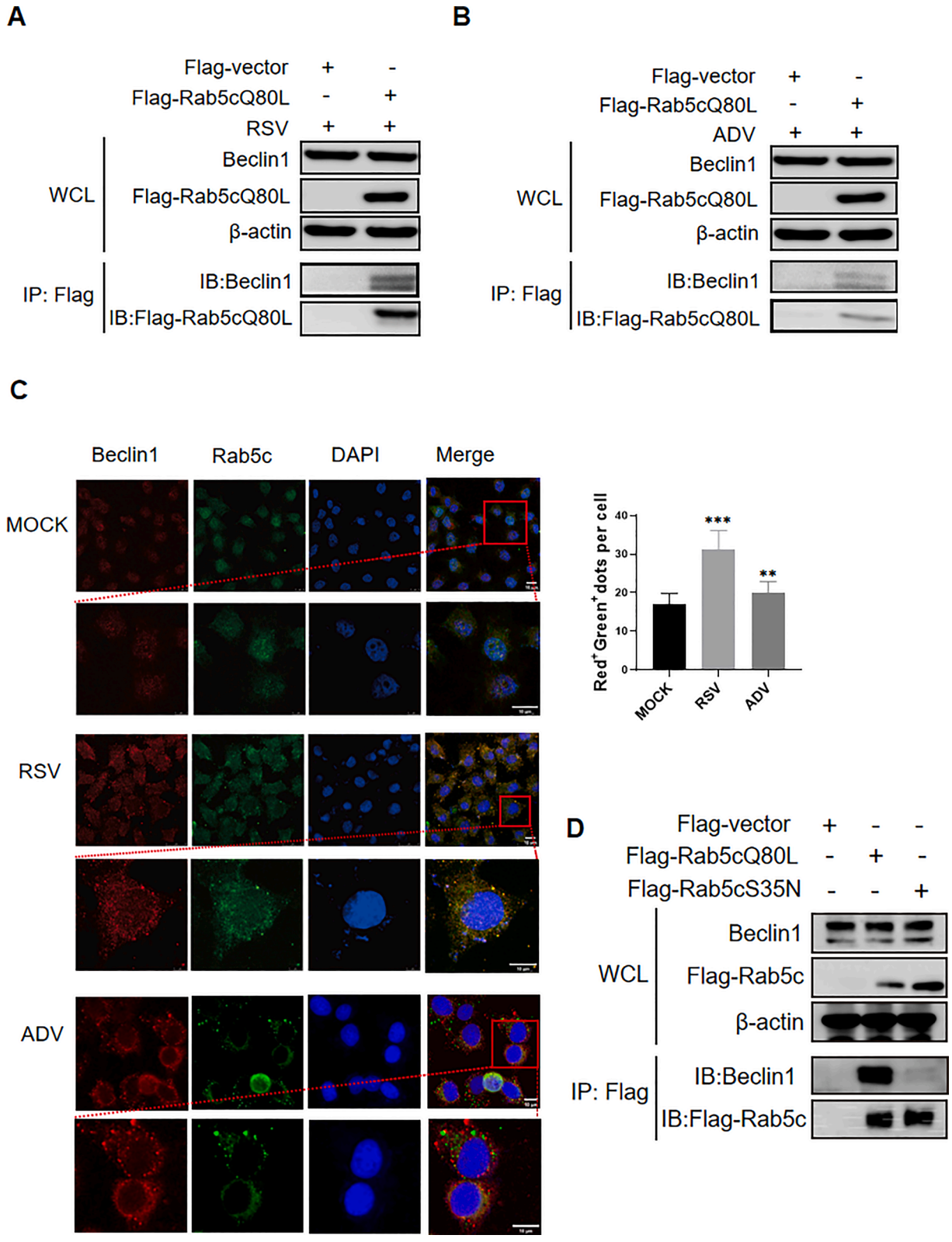

Fig. 4. Rab5c can interact with autophagy key protein Beclin1.
(A and B) HEK 293 cells were transfected with Flag empty vector / Flag-Rab5c Q80L plasmid for 24 h and then were infected with RSV (A) or ADV (B) for 24 h. The cell lysate was immunoprecipitated with anti-Flag and blotted with anti-Flag and anti-Beclin1 antibodies.
(C) A549 cells were infected with RSV or ADV for 24 h immuno-labeled with anti-Beclin1 and anti-Rab5c. A laser confocal microscope was used to observe the colocalization of Beclin1 and Rab5c. Nuclei were stained with DAPI. Scale bar=10 μm.
(D) HEK 293 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h. The cell lysate was immunoprecipitated with anti-Flag and blotted with anti-Flag and anti-Beclin1 antibodies.
(***$P<0.001$, **$P<0.01$,*$P<0.05$).

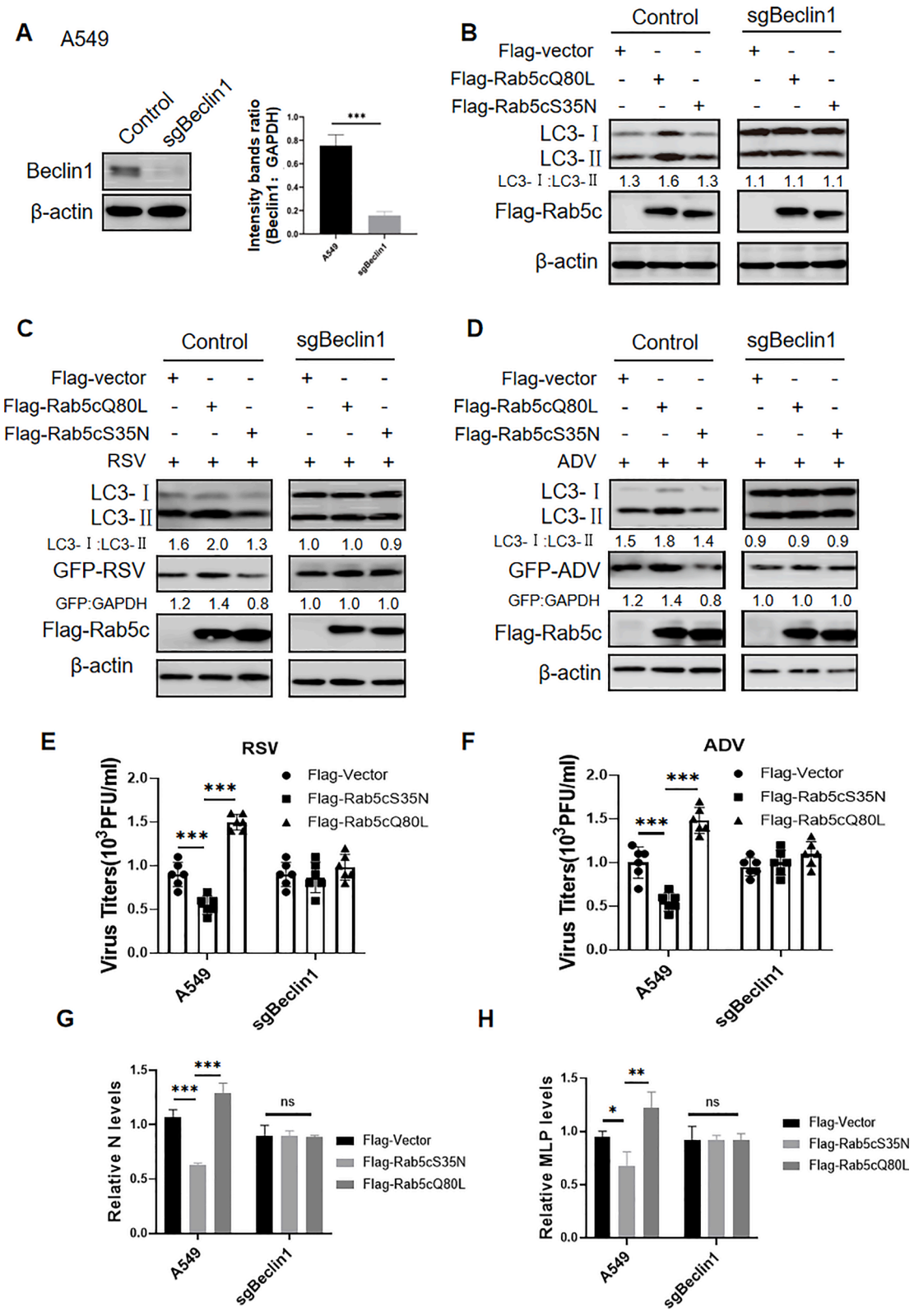

Fig. 5. Rab5c can promote RSV and ADV replication by interacting with Beclin1.
(A) Stable Beclin1 knockdown A549 cells or Negative Control A549 cells were generated using the CRISPR/Cas9 system.
(B–H) Control or sgBeclin1 cells were transfected with Flag-Rab5c Q80L or Flag-Rab5c S35N plasmids for 24 h and then were infected with GFP-RSV or GFP-ADV for 24 h. The expression levels of LC3-II and GFP proteins were detected by Western blot (B-D). The total viral titers were detected by viral plaque assay (E and F). The relative expression levels of RSV N (G) and ADV MLP (H) were detected by qRT-PCR.
(***P<0.001, **P<0.01, *P<0.05).

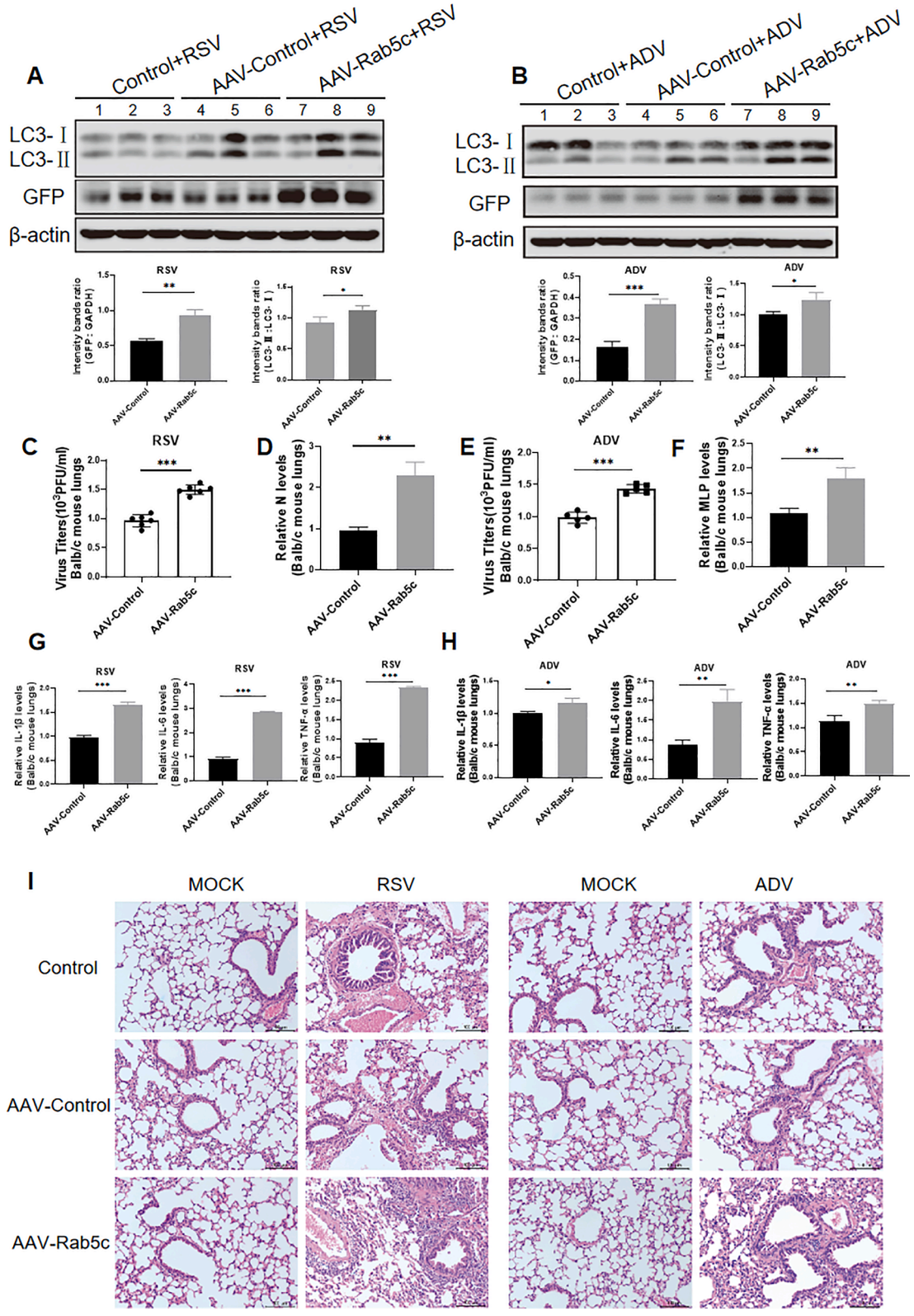

Fig. 6. Rab5c induces autophagy and promotes RSV and ADV replication *in vivo*.
(A-F) Balb/c mice were infected with AAV-Control or AAV-Rab5c, followed by infection with RSV or ADV. Mouse lungs were harvested, and the expression of LC3II, GFP and β-Actin proteins was analyzed with immunoblotting with specific antibodies (A and B). The total viral titers were detected by viral plaque assay (C and E). The relative expression levels of RSV N (D) and ADV MLP (F) were detected by qRT-PCR.
(G and H) Balb/c mice were treated as described in panel (A-F). The relative expression levels of IL-1β, IL-6, and TNF-α were detected by qRT-PCR.
(I) Balb/c mice were treated as described in panel (A-F). Lung pathology was detected by H&E stain. Scale bar=100 μm.
(***$P<0.001$, **$P<0.01$, *$P<0.05$).

RSV or ADV infected A549 cells. To investigate whether Rab5c affects virus replication through endocytosis, we transfected virus-infected cells with the plasmids of activated Rab5c or inactivated Rab5c, and found that the activated or inactivated Rab5c does not affect viral gene expressions in early stage of virus infection. It was suggesting that Rab5c activity does not affect viral endocytosis. Totally, endocytosis has no effect on Rab5c expression and Rab5c activity does not affect virus entry.

Rab5 plays an important role in the regulation of autophagy. Studies have shown that Rab5 is essential for the recruitment of Rab7 to PI3P-positive autophagosomes, and the recruitment relies on Ccz1-Mon1. Loss of Rab5 disrupts subsequent steps of autophagy (Hegedűs et al., 2016). Autophagy, a highly conserved cellular process, plays a significant role in viral infections (Chen et al., 2023; White et al., 2023). Many viral infections are accompanied by the initiation of autophagy (Dong and Levine, 2013; Kim et al., 2010). Autophagy plays a complex role in infections: on one hand, the host cell employs autophagy to engulf and degrade viruses; on the other hand, viruses have evolved mechanisms to evade autophagic degradation and even exploit autophagy to promote their own replication (Choi et al., 2018; Paul and Münz, 2016). It has been reported that SARS-CoV-2, the novel coronavirus, can induce and exploit cellular autophagy. The viral ORF7a protein cleaves SNAP29, leading to downregulation of SNAP29 expression and disruption of normal fusion between autophagosomes and lysosomes, thus inducing incomplete autophagy to facilitate viral replication (Corona and Jackson, 2018; Hou et al., 2023). Influenza A virus can disrupt autophagy by directly interacting with the autophagy protein LC3 through its M2 protein (Beale et al., 2014). Human parainfluenza virus (HPIV) M protein interacts with TUFM on mitochondria, recruiting LC3 to form autophagosomes and promoting virus replication (Beale et al., 2014; Song et al., 2020). Our previous study has demonstrated that RSV can induce cellular autophagy through the ROS-dependent AMPK-mTOR pathway, thereby inhibiting apoptosis and facilitating virus replication (Li et al., 2018). In this study, we also demonstrated that ADV can induce autophagy. Besides, we found that the activated Rab5c upregulates the level of LC3-II, while the inactivated Rab5c downregulates the level of LC3-II. The effect of Rab5c on viral replication can be partially reversed by autophagy inhibitors. Importantly, we discovered that activated Rab5c can interact with Beclin-1 in A549 cells.

Our study has revealed that Rab5c can promote the replication of RSV and ADV through autophagy, but further research is still needed to enrich our understanding on the mechanisms of other respiratory viruses replication such as influenza virus and parainfluenza virus. Additionally, the partial reversal of the impact of Rab5c on viral replication by autophagy inhibitors suggests that Rab5c may influence virus replication through other mechanisms. One study indicates that Rab5 can inhibit the production of IRF1-dependent IFN-λ, thereby affecting virus replication (Mo et al., 2021). In exploring the relationship between endocytosis and NRAV/Rab5c, it is important to employ various techniques, such as qRT-PCR, to elucidate this relationship from different perspectives. In the future, more direct and visual methods for monitoring the viral lifecycle, such as single-particle imaging analysis, can be applied to further refine our understanding of the relationship between endocytosis and Rab5c (Mo et al., 2021).

Our research group has conducted a series of studies on the interaction between RSV infection and host cells. Previous results showed that RSV leads to a decreased expression of Lnc NRAV, which, through molecular sponging, reduces the binding of miR-509–3p. As a result, there is an increase in the binding of miR-509–3p to Rab5c mRNA, leading to a decrease in Rab5c expression (Li et al., 2020). Additionally, RSV can induce cellular autophagy through the ROS-dependent AMPK-mTOR pathway, thereby inhibiting apoptosis and facilitating virus replication (Li et al., 2018). In our experiments, we have gained preliminary insights into the role of Rab5c in respiratory virus replication. Our results suggested that Rab5c can promote respiratory virus replication by inducing autophagy. It provides a new perspective for exploring the relationship between viruses and Rab5c, and offers new avenues for the development of potential antiviral targets.

Declaration of ethics statement

The animal study was approved by the Research Ethics Committee of the Second Hospital of Hebei Medical University with the permit number 2023-AE311 (Shijiazhuang, China).

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CRediT authorship contribution statement

Xiuli Wang: Methodology, Validation, Investigation, Resources. **Jing Cheng:** Data curation, Writing – review & editing, Visualization. **Linchao Shen:** Conceptualization, Formal analysis. **Meixi Chen:** Formal analysis, Validation. **Keran Sun:** Methodology, Writing – original draft. **Jian Li:** Conceptualization, Methodology. **Miao Li:** Writing – review & editing, Supervision. **Cuiqing Ma:** Conceptualization, Resources. **Lin Wei:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Supplementary materials

Supplemental Figure 1 ADV and RSV induce autophagy.

(A and B) A549 cells were infected with RSV (A) or ADV (B) for 24 h, 36 h, and 48 h. The expression levels of LC3-II and P62 proteins were detected by Western blot.

Supplemental Figure 2 The overexpression of Rab5c is validated *in vivo*.

Balb/c mice were infected with AAV-Control or AAV-Rab5c, followed by infection with RSV or ADV. Mice were sacrificed to measure the lung's Rab5c protein expression by immunoblotting (A) and immunofluorescence (B).

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2024.199324.

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