Pseudorabies virus hijacks Rab6 protein to promote viral assembly

Dong-Ge Liang
Henan Agricultural University

Shi-Bo Zhao
Henan Agricultural University

Guo-Yu Yang
Ministry of Agriculture and Rural Affairs of the People's Republic of China

Ying-Qian Han
Henan Agricultural University

Bei-Bei Chu
Henan Agricultural University  https://orcid.org/0000-0003-2961-4754

Sheng-Li Ming (✉ mingsl911102@163.com)
Henan Agricultural University  https://orcid.org/0000-0002-7505-6143

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Abstract

Pseudorabies virus (PRV) is the causative agent of Aujeszky's disease, also known as pseudorabies, in pigs. Rab6, a small GTPase, is thought to be essential for certain membrane trafficking activities and is associated with the regulation of exocytosis. Whether Rab6 is involved in PRV infection has not been previously reported. We found that the mRNA and protein levels of Rab6 were significantly upregulated in PK-15 cells and porcine alveolar macrophages, as well as in the lungs and spleen of PRV-infected mice. Overexpression of the wild-type and GTP-bound mutant of Rab6 promoted PRV proliferation, while the GDP-bound mutant inhibited it, suggesting that the GTPase activity of Rab6 is critical for PRV propagation. We further confirmed that knockdown of Rab6 inhibited PRV proliferation. Additionally, our results demonstrated that Rab6 knockdown disrupted PRV assembly. Mechanistically, Rab6 interacted with PRV glycoproteins gB and gE, which are crucial for PRV assembly. Our data suggest that PRV co-opts Rab6 to facilitate viral assembly and provide a potential new therapeutic target for PRV infection.

Introduction

Pseudorabies virus (PRV) is an alphaherpesviruses [1] The genome of PRV is approximately 143 kb and encodes at least 70 open reading frames [2]. PRV is highly adaptive, rapidly spreading, and has a high mortality rate. It affects pigs' respiratory, nervous, and reproductive systems, and can be highly damaging, often resulting in the extermination of entire herds, thereby posing a severe threat to the pig farming industry. [3]. Apart from pigs, PRV can also infect a variety of livestock and humans [4]. Recent studies have found that PRV may lead to endophthalmitis and encephalitis in humans [5–7]. These findings suggest that PRV infection is a potential public health risk that is not limited to the pig industry. Consequently, it is essential to develop novel strategies to prevent PRV infection.

Rab proteins, a large family of small GTPases, play a significant role in the cell's internal machinery, orchestrating a diverse array of cellular processes, predominantly related to vesicle trafficking [8, 9]. They are vital for a range of biological functions, including immune responses, cell growth, differentiation, and signal transduction [10–13]. More than 60 members of the Rab family have been identified, each characterized by a conserved molecular weight of approximately 21–25 kDa. Like other GTPases, Rab proteins generally exist in either an inactive GDP-bound state or an active GTP-bound state [14, 15]. These states are regulated by specific GTPase-activating protein and guanine nucleotide exchange factors [16, 17]. Enveloped viruses typically depend on the host's vesicle transport mechanisms to direct their structural proteins and genomes to the sites of virus replication, assembly, and budding. Rab GTPases are critical for the replication of many viral pathogens that cause human diseases [18]. For viruses to proliferate, they must enter host cells, replicate their genomes, assemble new virions, and then exit the host cell. Viruses often exploit Rab proteins to move their components within host cells, a process essential for each stage of their lifecycle [19]. For instance, Rab11-mediated recycling endosome transport is crucial for the assembly and release of new viral particles, as seen in the case of influenza [20].
Rab6 belongs to the Rab family, a group of small GTPases, and is essential for the successful functioning of cells. It is responsible for the budding, transport, and anchoring of vesicles within cells [21]. Regarding the association between Rab6 and viruses, numerous studies suggest that several viruses interact with Rab family proteins to manipulate the host's cellular mechanisms for their own replication. Specifically, Rab6 has been implicated in various stages of the viral life cycle [19, 22, 23]. For instance, Rab6 is known to participate in the intracellular transport of viral particles during herpes simplex virus type 1 (HSV-1) infection [24]. As an important member of the Rab family of small GTPases, Rab6 is likely to be involved in PRV infection due to its critical role in vesicle trafficking [25, 26]. Any virus that requires intracellular transport and replication within host cells, such as PRV, could potentially exploit Rab6 for these processes to ensure its propagation.

The replication of PRV and its subsequent exit from the cell is a complex process that requires effective use of the cell's machinery [27]. In this study, we examined the effects of Rab6 on PRV replication. We found that PRV hijacked Rab6 to facilitate viral assembly. This underscores the significance of Rab6 in PRV infection and suggests new possibilities for the development of the effective antiviral therapies.

Materials and methods

Reagents

TRIzol Reagent (D9108B) and SYBR Premix *Ex Taq* (RR420A) were ordered from TaKaRa; anti-Rab6 (10187-2-AP), anti-HA (51064-2-AP), anti-FLAG (66008-4-Ig) were purchased from Proteintech. Antiserum against PRV glycoprotein gB and gE were generated by immunizing mice with purified recombinant gB and gE.

Cells, viruses and plasmids

Porcine kidney epithelial PK-15 cells (CCL-33, ATCC), PAMs [28], HEK293T (CRL-11268, ATCC) cells were grown at 37°C with 5% CO2 in DMEM (12110, Solarbio) supplemented with 10% fetal bovine serum (FBS, A5669701, Gibco), contained 100 U/mL penicillin and 100 µg/mL streptomycin sulfate (B540732, Sangon).

The virulent PRV isolate QXX (PRV-QXX) was kindly donated by Yong-Tao Li from the college of Veterinary Medicine, Hennan Agricultural University [29]. The recombinant PRV strain of PRV-GFP, derived from the PRV Hubei strain with the TK gene replaced by a GFP expression cassette from the pEGFP-N1 plasmid [30].

Full-length porcine Rab6 cDNA was amplified by polymerase chain reaction (PCR). Rab6 cDNA was cloned into p3×FLAG-CMV-10 expression plasmids to generate FLAG-Rab6, FLAG-Rab6 Q72L and FLAG-Rab6 T27N. All plasmids were transfected with Lipofectamine 3000 (L3000015, Invitrogen) according to the manufacturer's instructions. The primer sequences used for gene amplification were used as follows: Rab6-Fw: 5’-ATGTCCACGGCGGAGAC-3’, Rab6-Rv: 5’-TTAGCAGGAAACAGCCTCTTCA-3’, Rab6 Q72L-Fw:
5′-AGGTCTAGAGCGGTTCAGGAGCTTGATTCCTA-3′, Rab6 Q72L-Rv: 5′-TGAACCGCTCTAGACCTGCTGTGTCCCATAATTGC-3′; Rab6 T27N-Fw: 5′-GCGTTGGAAAGAACTCTTTGATCACCAGATTCATGTATGA-3′, Rab6 T27N-Rv: 5′-AAGAGTTCTTCTCAACGCTTTGCTCCCCAGGA-3′.

**Mice**

Female 6- to 8-week-old C57BL/6J mice were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China) and maintained in a specific pathogen-free animal facility according to the Guide for the Care and Use of Laboratory Animals and the related ethical regulations instilled at Henan Agricultural University.

**Cells viability analysis**

Cell viability was assessed using a Cell Counting Kit-8 (CCK-8, GK3607, DingGuo). Cells were seeded in 96-well plates at a density of 1×10⁴ per well for 24 h. The next day, CCK-8 solution (10 µL) was added to each well, followed by incubation at 37°C for 3 h. The absorbance was then detected at 450 nm with a microplate reader (Varioskan Flash, Thermo Fisher Scientific).

**Immunoblotting analysis**

The cells were lysed in RIPA buffer (P0013B, Beyotime Biotechnology) supplemented with a protease and phosphatase inhibitor cocktail (HY-K0010 and HY-K0022, MedChemExpress). The concentration of protein was determined using the BCA Protein Assay Kit (BCA01, DingGuo). The protein samples were separated by SDS-PAGE and transferred to a membrane. The membrane was incubated with 5% skim milk (A600669, Sangon) powder for 1 h at room temperature. It was then incubated with the primary antibody overnight at 4°C, followed by incubation with proper horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Luminata Crescendo Western HRP substrate (WBLUR0500, Millipore) was used to visualize the immunoblotting results on the GE AI600 imaging system.

**qRT-PCR analysis**

Total RNA was extracted using TRIzol Reagent (TaKaRa) and reverse-transcribed with a PrimeScript RT reagent kit (RR047A, TaKaRa). qRT-PCR was performed on the QuantStudio 6 Flex Real-Time PCR System using SYBR Premix ExTaq (TaKaRa). The data were normalized to the expression of the control gene ACTB. Transcripts were quantified using the 2⁻^ΔΔCt method. The following primers were used for qRT-PCR analysis: porcine ACTB-Fw: 5′-GCACAGAGCCTCGCCTT-3′, porcine ACTB-Rv: 5′-CCTTGCACATGCCGGAG-3′; PRV gH-Fw: 5′-CTCGCCATCGTCAGCAA-3′, PRV gH-Rv: 5′-GCTGCTCCTCCATGTCCA-3′; porcine RAB6-Fw: 5′-GAGCGTACACCTAACCACCC-3′, porcine RAB6-Rv: 5′-GCGTTGGAAAGAACTCTTTGATCACCAGATTCATGTATGA-3′; mouse ACTB-Fw: 5′-CCCCATTGAACATGGCATTG-3′, mouse ACTB-Rv: 5′-ACG ACCAGGCCATACAGG-3′; mouse Rab6-Fw: 5′-GACGTCTTCTCCATCATCAGGATGTA-3′.

**Immunofluorescence analysis**
Cells grown on climbing tablets were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. The cells were then permeabilized with 0.1% Triton X-100 for 10 min. Afterward, they were incubated with PBS containing 10% FBS for 1 h at room temperature. The cells were washed with PBS and then incubated with the appropriate Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. Finally, the cells were mounted in ProLong Diamond with DAPI (P36971, Invitrogen). Images were captured using a Zeiss LSM 800 confocal microscope.

RNA interference

Short hairpin RNAs (shcontrol: 5’-GCCACAACGTCTATATCATGG-3’; shRab6-1: 5’-GGAGCTTGTAGCTAGCTACA-3’; shRab6-2: 5’-GCTTGATTCTAGCTACATTC-3’; shRab6-3: 5’-GCTACATTCTGACTCCACTG-3’) were synthesized as double-stranded oligonucleotides and cloned into the pLKO.1 vector and cotransfected with packaging plasmids pMD2.G (12259, Addgene) and psPAX2 (12260, Addgene) into HE293T cells. Lentiviruses were harvested at 48 h post-transfection and used to infect cells that were then selected with puromycin (4 µg/mL) for 7 days. Knockdown efficiency was determined by qRT-PCR or immunoblotting analysis.

Plaque assay

Serially diluted PRV-QXX samples were inoculated into Vero cells grown in 12-well plates. After 3–5 days of incubation, when cytopathic effects were observed at about 80%, the cells were stained with 1% crystal violet. The dye solution was then rinsed away with distilled water, and the number of plaques (empty spots) was calculated.

The 50% tissue culture infective dose (TCID\text{50}) assay

On Day 0, Vero cells were seeded in a 96-well plate at 1×10^4 cells per well. On Day 1, the cells were inoculated with serially diluted viruses (ranging from 10^{-1} to 10^{-12} dilutions) for 1 hour at 37°C. The excess viral inoculum was removed by washing with PBS. Then, 200 µL of maintenance medium (DMEM with 2% FBS) was added to each well and the cells were cultured for 3–5 days. The cells displaying the expected cytopathic effect were observed daily, and the TCID\text{50} value was calculated using the Reed–Muench method.

Viral attachment assay

Cells were incubated with PRV-QXX (MOI = 10). at 4°C for 2 h. After three extensive washes with ice-cold phosphate-buffered saline (PBS), viral attachment assay was performed using qRT-PCR analysis of PRV genome copy numbers on the PM.

Viral entry assay

Cells were incubated with PRV PRV-QXX (MOI = 10) at 4°C for 2 h. Then, the cells were extensively washed with ice-cold PBS three times and incubated at 37°C for 10 min to allow entry. After washing with trypsin (1 mg/mL) to remove the residual virions on the PM, viral entry was detected via qRT-PCR analysis of viral genome copy numbers in the cells.
Viral assembly assay

Infect PK-15 cells with PRV-QXX (MOI = 5). The assembly efficiency of the virus in the supernatant was determined by comparing the infection titer (TCID$_{50}$/mL) with the total genomic equivalent of PRV (gE).

Co-IP assay

Cells were harvested and lysed in 1 mL of lysis buffer (PBS/1% NP-40, 5 mM EDTA, 5 mM EGTA) and clarified by centrifugation at 16,000 $g$ for 10 min at 4°C. Next, 900 µL aliquots were incubated with 40 µL of a 1:1 slurry of Sepharose conjugated with either IgG (17-0969-01, GE Healthcare) for 1 h at 4°C. The beads were washed three times with lysis buffer and eluted with SDS sample buffer by boiling for 10 min before immunoblotting.

Statistical analysis

Data are representative of at least three independent experiments for quantitative analysis and expressed as the means ± standard errors of the means. All data were analyzed in Prism 7 software (GraphPad Software, Inc) with two-tailed Student’s $t$-test, and $P < 0.05$ was considered statistically significant.

Results

PRV infection upregulates Rab6 expression.

To determine the role of Rab6 in PRV infection, we evaluated the expression of Rab6 following a PRV challenge in vitro. Cells were infected with PRV-QXX for durations ranging from 0 to 24 hours, and both the mRNA and protein levels of Rab6 in the cells were measured. PRV infection resulted in increased Rab6 mRNA expression in both PK-15 and porcine alveolar macrophage (PAM) cells (Figs. 1A and B). Consistent with the mRNA levels, Rab6 protein levels in PK-15 and PAM cells were also elevated following PRV infection (Figs. 1C and D). Next, we investigated whether PRV upregulated the expression of Rab6 in vivo. Mice were either mock-infected or intranasally infected with PRV-QXX for three days. The lungs and spleens were assessed for Rab6 mRNA levels by quantitative real-time polymerase chain reaction (qRT-PCR), and the lungs were assessed for Rab6 protein levels by immunoblotting analysis. PRV infection resulted in elevated Rab6 expression in the tissues of infected mice compared with those of mock-infected mice (Figs. 1E–G). These results suggest that PRV promotes the expression of Rab6 in vitro and in vivo.

Rab6 overexpression promotes PRV infection.

To examine the role of Rab6 in PRV proliferation, we constructed a plasmid encoding FLAG-tagged Rab6. PK-15 cells were transfected with different concentrations of the FLAG-Rab6 plasmid or an empty vector for 24 hours, followed by infection with PRV-GFP. Fluorescent microscopy and fluorescence intensity analysis revealed a significant enhancement of the GFP fluorescence signal after overexpression of
FLAG-Rab6, suggesting that Rab6 overexpression promoted PRV-GFP infection (Figs. 2A and B). We also examined the effect of Rab6 overexpression on PRV gB expression. Immunoblotting analysis showed a significant increase in PRV gB expression due to overexpression of FLAG-Rab6 (Fig. 2C). The plaque assay indicated that overexpression of Rab6 enhanced the production of viable progeny (Fig. 2D). Additionally, we constructed the GTP-bound mutant Rab6 Q72L (constitutively active) and the GDP-bound mutant Rab6 T27N (dominant negative). PK-15 cells were transfected with vector, FLAG-Rab6 Q72L and FLAG-Rab6 T27N. The overexpression of Rab6 Q72L was found to significantly elevate the expression of PRV gB, whereas the overexpression of Rab6 T27N had no discernible effect on the expression of PRV gB, as demonstrated by Western blot analysis (Fig. 2E). In the plaque assay, it was observed that overexpression of Rab6 Q72L resulted in enhanced virus proliferation, while Rab6 T27N did not affect virus proliferation (Fig. 2F). These results suggest that the overexpression of Rab6 promotes PRV infection.

Rab6 knockdown inhibits PRV infection.

To further validate the role of Rab6 in PRV infection, we depleted Rab6 using short hairpin RNA (shRNA)-mediated RNA interference. Both qRT-PCR and immunoblotting analyses showed that these three Rab6-targeting shRNAs exhibited significant knockdown efficiency in PK-15 cells (Figs. 3A and B). In addition, the knockdown of Rab6 did not affect cell viability (Fig. 3C). Next, we infected shcontrol, shRab6-1, shRab6-2, and shRab6-3 PK-15 cells with PRV-GFP and assessed viral proliferation using fluorescence microscopy. The observed decrease in fluorescence intensity in cells with Rab6 knockdown, in comparison to control cells, suggests an inhibitory effect of Rab6 knockdown on PRV-GFP replication (Figs. 3D and E). To further assess viral replication, PRV gB protein levels were analyzed by immunoblotting, showing notably lower levels in Rab6-knockdown cells than in control cells (Fig. 3F). Additionally, when cells were infected with PRV-QXX, the viral titer was found to be lower in the shRab6-1, shRab6-2, and shRab6-3 cells compared to the shcontrol cells (Fig. 3G). Taken together, the data showed that Rab6 knockdown inhibited the proliferation of PRV.

Rab6 is involved in PRV assembly

To gain a more thorough understanding, we investigated the precise stages at which Rab6 affects PRV's life cycle. Viral attachment to host cells is the first step in a viral infection. Initially, we incubated shcontrol and shRab6-1 cells with PRV at 4°C for 2 hours. After extensive washing three times with ice-cold phosphate-buffered saline (PBS), viral attachment was detected by qRT-PCR analysis of viral genome copy numbers on the cells. Additionally, the cells were then shifted to 37°C for 10 minutes to allow entry. After washing with 1 mg/mL trypsin to remove residual virions on the plasma membrane (PM), viral entry was detected by qRT-PCR analysis of viral genome copy numbers in the cells. Our results demonstrated that the knockdown of Rab6 had no effect on PRV attachment and entry (Figs. 4A and B). We delved deeper into viral assembly. Following the knockdown of Rab6, virus assembly efficiency decreased, as indicated by the viral assembly results (Fig. 4C). Moreover, We further analyzed the infectivity of intracellular and extracellular PRV progeny virus by a TCID<sub>50</sub> assay. Both intracellular and extracellular
PRV titers were decreased at 2 h postinfection (Figs. 4D and E). To further ascertain this, we transfected PK-15 cells with vector, FLAG-Rab6, FLAG-Rab6 CA, FLAG-Rab6 DN, and an empty vector plasmid; Upon infecting the cells with the virus, the efficiency of virus assembly was determined. The results demonstrate that the overexpression of FLAG-Rab6 significantly boosts the assembly of PRV compared to transfection with an empty vector (Fig. 4F). Simultaneously, it was observed that overexpressing FLAG-Rab6 CA increased PRV virus assembly compared to the overexpression of FLAG-Rab6, while overexpressing FLAG-Rab6 DN showed no impact on PRV assembly compared to the empty vector group (Fig. 4F). In conclusion, Rab6 is involved in PRV assembly.

Rab6 interacts with PRV gB and gE.

Evidence has been presented that Rab6 can impact the assembly stages of the virus. It is commonly understood that the glycoprotein of the herpes virus is a critical element in the process of assembling and releasing progeny viruses [31]. The virus’s glycoprotein is encapsulated in the secondary envelope of vesicles derived from the trans-Golgi network, particularly during the assembly process of progeny viruses [32]. Initially, we conducted an analysis of the co-occurrence of PRV gB and gE in relation to Rab6. Our research demonstrated that when HA-tagged plasmids encoding gB and gE were expressed in PK-15 cells, the two proteins colocalized with Rab6 (Figs. 5A and B). Additionally, we transfected gl-HA, gM-HA, and gL-HA plasmids, and immunofluorescence detection showed that gl, gM, and gL did not colocalize with Rab6 (Figs. 5C-E). To validate the interaction between PRV gB and gE with Rab6, a co-immunoprecipitation (Co-IP) assay was performed on PK-15 cells that had been either mock-infected or infected with PRV. It was observed that PRV gB and PRV gE co-immunoprecipitated with Rab6, as illustrated in Figs. 5F and G. It appears that Rab6 is involved in the process of virus assembly through its interaction with PRV gB and gE.

Discussion

Host cells are essential for the completion of the life cycle of viruses [33]. Viruses are composed of either an RNA or DNA genome, protected by a protein capsid [34]. Certain viruses possess a lipid bilayer derived from the cellular membranes of their host. To replicate, viruses must exploit the host cell’s systems to produce new RNA, DNA, proteins, and lipid envelopes [35, 36]. Virus life cycles may be distinct, yet they all share five steps: infiltration of the cell, translation of viral proteins, replication of the genome, assembly of the viral particle, and exit from the cell [37]. Enveloped viruses typically rely on the host’s vesicular trafficking pathways to transport their structural proteins and genetic material to areas where they can replicate, assemble, and bud [18]. The Rab GTPases, the largest family of small GTPases, include close to 70 members involved in vesicular transport [38]. The Golgi Rab group is largely comprised of the Rab6 protein, which epitomizes the Rab6 subfamily [39, 40]. It is evident that the Rab family plays a pivotal role in the process of viral infection. The Golgi apparatus, situated at the core of the animal cell's internal membrane system, plays a significant role in the post-translational modification of proteins and the organization of secretory pathway transport [41]. Rab6 is a critical protein of the Rab family, involved in the retrograde movement within the Golgi apparatus [41]. This study demonstrates the significance of
Rab6 in PRV infection, acting as a co-factor to facilitate virus assembly, thereby contributing to the replication of PRV.

Results of investigations have confirmed that Rab proteins and their effector components are essential for the growth of viruses. For example, Rab2 has been observed to promote the proliferation of the classical swine fever virus [42], and Rab11a has the ability to boost the replication of PRRSV in the host organism by managing the autophagy process [43]. Our results demonstrated that PRV infection was linked to an increase in Rab6 expression and that when Rab6 was overexpressed, it enhanced PRV proliferation. Conversely, if Rab6 was knocked down, the proliferation of PRV was inhibited. To investigate the effect of Rab6 on the proliferation of PRV, we examined the life cycle of PRV, although the exact stage at which Rab6 influences the cycle remains unknown. Hollinshead and Raza conducted knockdown experiments on certain Rab proteins and determined that Rab5 and Rab11 are involved in the entry of the virus into epithelial cells through the endocytic pathway after HSV infection [19, 44]. Our research established that the knockdown of Rab6 had no effect on the adsorption and entry of PRV; however, the assembly test of the virus revealed that the knockdown of Rab6 inhibited the assembly of PRV. Additionally, when Rab6 was overexpressed in PK-15 cells, it was observed that Rab6 overexpression facilitated the assembly of PRV. These outcomes indicate that Rab6 plays a significant role in the assembly process of PRV.

The exact molecular processes that PRV uses to exploit the cell's transportation system during morphogenesis remain unclear. Specific details regarding the secondary envelopment and exit of the virus still require further exploration. The final stages of viral assembly involve the budding of trans-Golgi network-derived vesicles coated with viral glycoproteins and tegument proteins, an event known as secondary envelopment [45, 46]. Recently, many investigations have provided mounting evidence for the utilization of Rab proteins by enveloped viruses for their morphogenesis and egress pathways. For instance, research has shown that both influenza A and respiratory syncytial virus employ the Rab11a pathway for enveloping and budding [47], while Rab27a affects HSV-1 viral assembly by interacting with the glycoprotein gB [48]. Consequently, we investigated whether viral glycoproteins were associated with Rab6. Our observations revealed that Rab6 was associated with PRV gB and gE; however, it was not co-localized with gM, gI, and gL. The Co-IP assay confirmed that Rab6 interacted with both gB and gE. In summary, the findings of our research suggest that Rab6 could be a beneficial host factor in PRV replication and thus could be a valuable target for the development of drugs and vaccines to inhibit PRV replication.

**Declarations**

**Ethics approval and consent to participate**

The Animal Care Committee of Henan Agricultural University (Zhengzhou, People's Republic of China) approved this study (approval number 11–0085), and all animals were maintained in a specific...
pathogen-free animal facility according to the related ethical regulations at Henan Agricultural University and the guide for the care and use of laboratory animals.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Writing review and supervision, data curation, writing original draft: DL; formal analysis, software, visualization, investigation: BZ; resources: GY; project administration, supervision: YH; funding acquisition, supervision, writing—review and editing: BC and SM. All authors have read and approved the final manuscript.

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**Availability of data and materials**

All data and materials generated for this study are included in the article.

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Figures
Figure 1

PRV infection stimulates Rab6 expression.

(A and B) PK-15 and PAMs cells were infected with PRV-QXX (MOI = 1) for 24 h. The mRNA levels of Rab6 were evaluated by qRT-PCR analysis. **P < 0.01, ***P < 0.001.

(C and D) PK-15 and PAMs cells were infected with PRV-QXX (MOI = 1) for 24 h. The protein levels of Rab6 and PRV gB were evaluated by immunoblotting analysis.

(E and F) Mice were either mock-infected or intranasally infected with PRV-QXX (5×10³ TCID₅₀/50 µL per mouse) for 3 days. The mRNA levels of Rab6 in the lungs and spleen were analyzed by qRT-PCR analysis (n = 3 per group). ***P < 0.001.

(G) Mice were treated as described in E). The protein levels of Rab6 and PRV gB in the lungs was assessed by immunoblotting analysis (n = 3 per group).
Figure 2

Rab6 overexpression promotes PRV infection.

(A) PK-15 cells were transfected with the p3×FLAG-CMV-10 (Vector) or FLAG-Rab6 plasmid (0–2 μg) for 12 hours and then infected with PRV-GFP (MOI = 0.001) for 24 h. Viral replication was analyzed by fluorescence microscopy. Scale bar: 200 μm.

(B) ImageJ analyzed the relative fluorescence intensity in A. *P < 0.05, **P < 0.01.

(C) PK-15 cells were transfected with the p3×FLAG-CMV-10 (Vector) or FLAG-Rab6 plasmid (0–8 μg) for 12 hours and then infected with PRV-QXX (MOI = 1) for 24 h. The protein levels of PRV gB and FLAG-Rab6 were assessed by immunoblotting analysis.

(D) PK-15 cells were transfected with the p3×FLAG-CMV-10 or FLAG-Rab6 plasmid (0–2 μg) for 12 hours and then infected with PRV-QXX (MOI = 1) for 24 h. Viral titers were determined by the plaque assay. *P < 0.05, **P < 0.01, ***P < 0.001.

(E) PK-15 cells were transfected with FLAG-CMV-10 (Vector) FLAG-Rab6, FLAG-Rab6 Q72L, and FLAG-Rab6 T27N plasmids for 12 h and then infected with PRV-QXX (MOI = 1) for 24 h. The protein levels of PRV gB and FLAG-Rab6 were assessed by immunoblotting analysis.

(F) PK-15 cells were transfected with p3×FLAG-CMV-10 (Vector), FLAG-Rab6, FLAG-Rab6 Q72L, and FLAG-Rab6 T27N plasmids for 12 h and then infected with PRV-QXX (MOI = 0.1 and 1) for 24 h. Viral
titers were determined by the plaque assay. *\(P < 0.05\), **\(P < 0.01\). ns, no significance.

**Figure 3**

**Rab6 knockdown inhibits PRV infection.**

(A and B) The levels of Rab6 mRNA and protein were assessed in shcontrol, shRab6-1, shRab6-2, and shRab6-3 PK-15 cells by qRT-PCR and immunoblotting analysis. *\(P < 0.05\), ***\(P < 0.001\).

(C) shcontrol, shRab6-1, shRab6-2, and shRab6-3 PK-15 cell viability was assessed by CCK-8 assay.

(D) shcontrol, shRab6-1, shRab6-2, and shRab6-3 PK-15 cells infected with PRV-GFP (MOI = 0.001) for 24 h were observed under a fluorescence microscope to analyze viral proliferation by GFP expression. Scale bar: 200 μm.

(E) ImageJ analyzed the relative fluorescence intensity in C. **\(P < 0.01\), ***\(P < 0.001\).

(F) shControl, shRab6-1, shRab6-2, and shRab6-3 PK-15 cells were infected with PRV-QXX (MOI = 1) for 24 h. The protein levels of PRV gB and Rab6 were assessed by immunoblotting analysis.

(G) shControl, shRab6-1, shRab6-2, and shRab6-3 PK-15 cells were infected with PRV-QXX (MOI = 1) for 24 h. Viral titers were determined by the plaque assay. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
Rab6 is involved in PRV assembly.

(A) shcontrol and shRab6-1 PK-15 cells were incubated with PRV (MOI = 10) at 4°C for 2 h. After the cells were washed three times with ice-cold PBS, viral attachment was assessed by qRT-PCR analysis of the genome copy numbers on the PM. ns, no significance.

(B) shcontrol and shRab6-1 PK-15 cells were incubated with PRV (MOI = 10) at 4°C for 2 h. Then, the cells were extensively washed with ice-cold PBS three times and incubated at 37°C for 10 min to allow entry. After washing with trypsin (1 mg/mL) to remove the residual virions on the PM, viral entry was detected via qRT-PCR analysis of viral genome copy numbers in the cells. ns, no significance.

(C) shcontrol and shRab6-1 PK-15 cells were infected with PRV (MOI = 5) for 24 h. The efficiency of viral assembly in the supernatants was determined by comparing the infectious titers (TCID$_{50}$ per milliliter) with the total PRV genome equivalents (GE). **$P < 0.01$.**
(D and E) shcontrol and shRab6-1 PK-15 cells were infected with PRV-QXX (MOI = 5). The extracellular (D) and intracellular (E) viruses were harvested and subjected to a TCID<sub>50</sub> assay to determine the viral titers at 2–24 hours post-infection (hpi). *P < 0.05.

(F) PK-15 cells were transfected with an p3×FLAG-CMV-10 (Vector), FLAG-Rab6, FLAG-Rab6 CA, and FLAG-Rab6 DN for 12 hours and then infected with PRV-QXX (MOI = 5) for 24 h. The efficiency of viral assembly in the supernatants was determined by comparing the infectious titers (TCID<sub>50</sub> per milliliter) with the total PRV genome equivalents (GE). ***P < 0.001. ns, no significance.

Figure 5

**Rab6 interacts with PRV gB and gE.**

(A–E) PK-15 cells were transfected with HA-tagged gB (A), gE (B), gl (C), gM (D) and gL (E) plasmids for 24 hours. The colocalization of Rab6 with gB, gE), gl, gM and gL was determined by immunofluorescence analysis. Scale bar: 10 μm The graphs depict the application of Image J software in conducting line scan analysis (A ~ E) on the image, and in analyzing the relative position of the two markers. Bars, 10 μm.

(F and G) PK-15 cells were transfected with an p3×FLAG-CMV-10 (Vector) and FLAG-Rab6 for 12 hours and then infected with PRV-QXX (MOI = 1) for 24 h. The interaction of Rab6 with gB (F) and gE (G) was analyzed by Co-IP analysis.