Supplementary Information

Rab27a regulates the transport of influenza virus membrane proteins to the plasma membrane

Tong Chen[1#], Aotian Ouyang[1#], Jiahui Zou[1], Yi Feng[1], Changsong Wu[1], Meijun Jiang[1], Shaoyu Tu[1], Ling Ding[1], Yanqing Cheng[1], Wenshuo Hu[1], Wei Sheng[1], Yanglin Li[1], Meilin Jin[1,2], Huanchun Chen[1,2,3,4], Hongbo Zhou[1,2,3,4]*

[#]These authors contributed equally to this work.

*Corresponding author: Email: hbzhou@mail.hzau.edu.cn (H.Z.)

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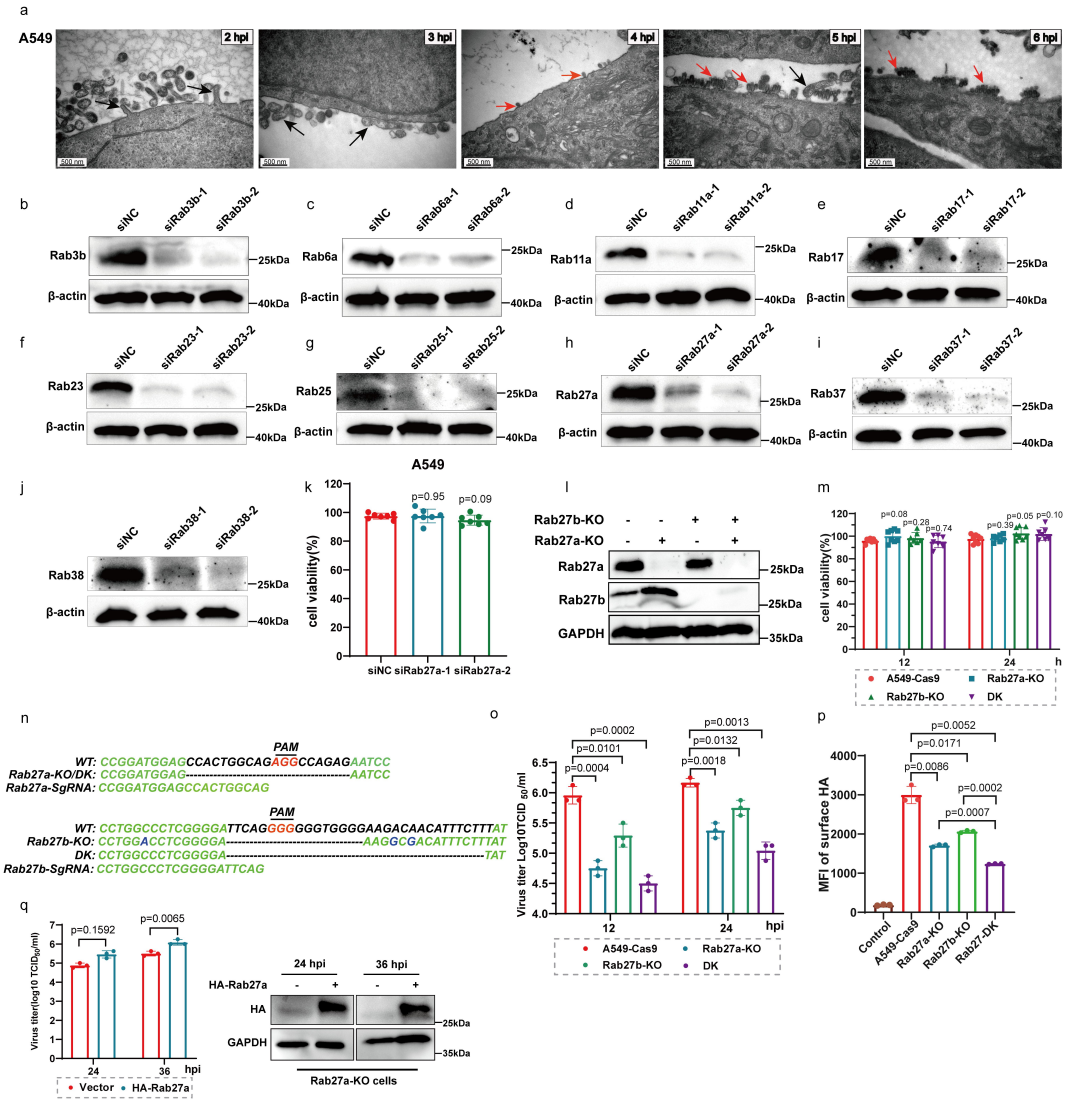
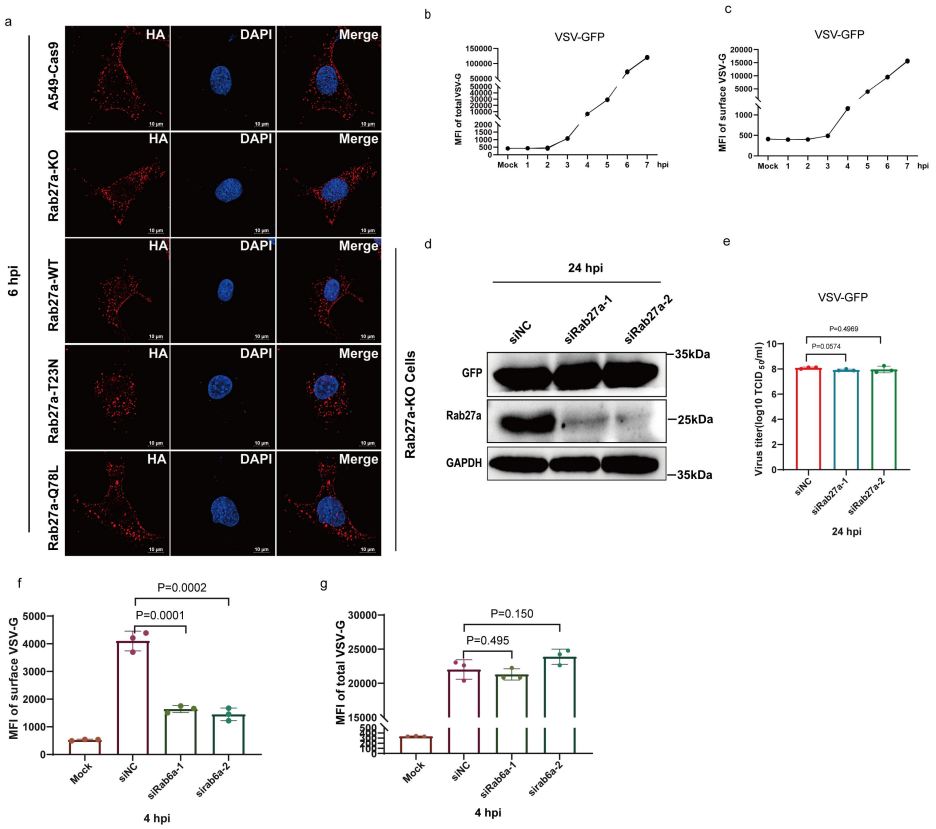


Supplementary Fig. 1. Rab27a promotes IAV replication

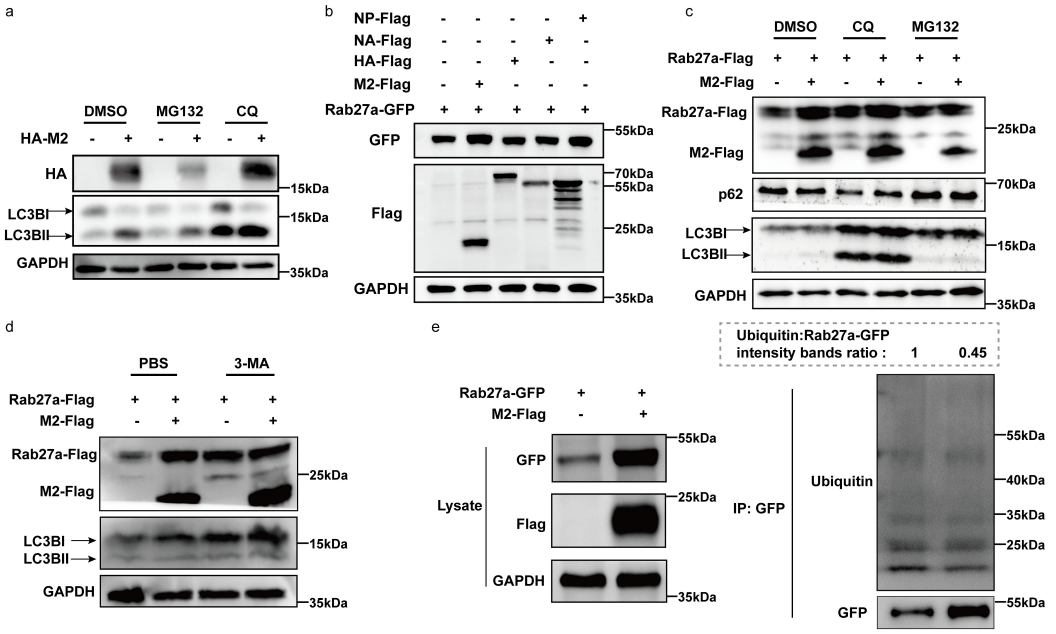
a A549 cells were infected with HM virus (MOI, 10). At 2, 3, 4, 5, 6 hpi, cells were fixed in glutaraldehyde, and influenza virus budding was observed by TEM. Black arrows indicate filamentous pseudopodia in cells, while red arrows indicate budding virus particles. Scale bar, 500 nm. Images shown are a representative field of vision from at least three independent experiments. **b-j** A549 cells were transfected with siNC, siRab3b (siRab3b-1, siRab3b-2), siRab6a (siRab6a-1,siRab6a-2), siRab11a (siRab11a-1, siRab11a-2), siRab17 (siRab17-1, siRab17-2), siRab23 (siRab23-1, siRab23-2), siRab25 (siRab25-1, siRab25-2), siRab27a (siRab27a-1, siRab27a-2), siRab37 (siRab37-1, siRab37-2), or siRab38 (siRab38-1, siRab38-2) for 24 h. Silencing efficiency was detected by western blot for each indicated protein, with β-actin serving as a loading control. **k** A549 cells were transfected with siNC,

siRAB27a-1, or siRab27a-2 for 24 h, then cell viability was measured by CCK-8 assay. **l** Rab27a and Rab27b protein levels in Rab27a-KO, Rab27b-KO, Rab27-DK, and A549-Cas9 cells were detected by western blot, with GAPDH serving as a loading control. **m** Rab27a-KO, Rab27b-KO, Rab27-DK, and A549-Cas9 cells were seeded in 96-well plates at approximately 50% confluence, and cell viability was assessed after 12 and 24 h using the CCK-8 assay. **n** Sequencing results from Rab27a-KO, Rab27b-KO, Rab27-DK and A549-Cas9 cells. **o, p** Rab27a-KO, Rab27b-KO, Rab27-DK and A549-Cas9 cells were infected with HM virus, viral titers of supernatants at 12 and 24 hpi were determined by $TCID_{50}$ assay on MDCK cells in (**o**), cell surface levels of HA protein at 6 hpi were quantified by flow cytometry in (**p**) (MOI, 10). **q** Rab27a-KO cells were transfected with Rab27a synonymous mutation plasmid or empty vector for 24 h, followed by infection with HM virus (MOI, 0.1). At 12 and 24 hpi, protein levels were detected by western blot, and viral titers in supernatants were determined by $TCID_{50}$ assay on MDCK cells. Data are presented as mean ± SD from three independent experiments. Statistical significance was assessed using a two-tailed Student's t-test.



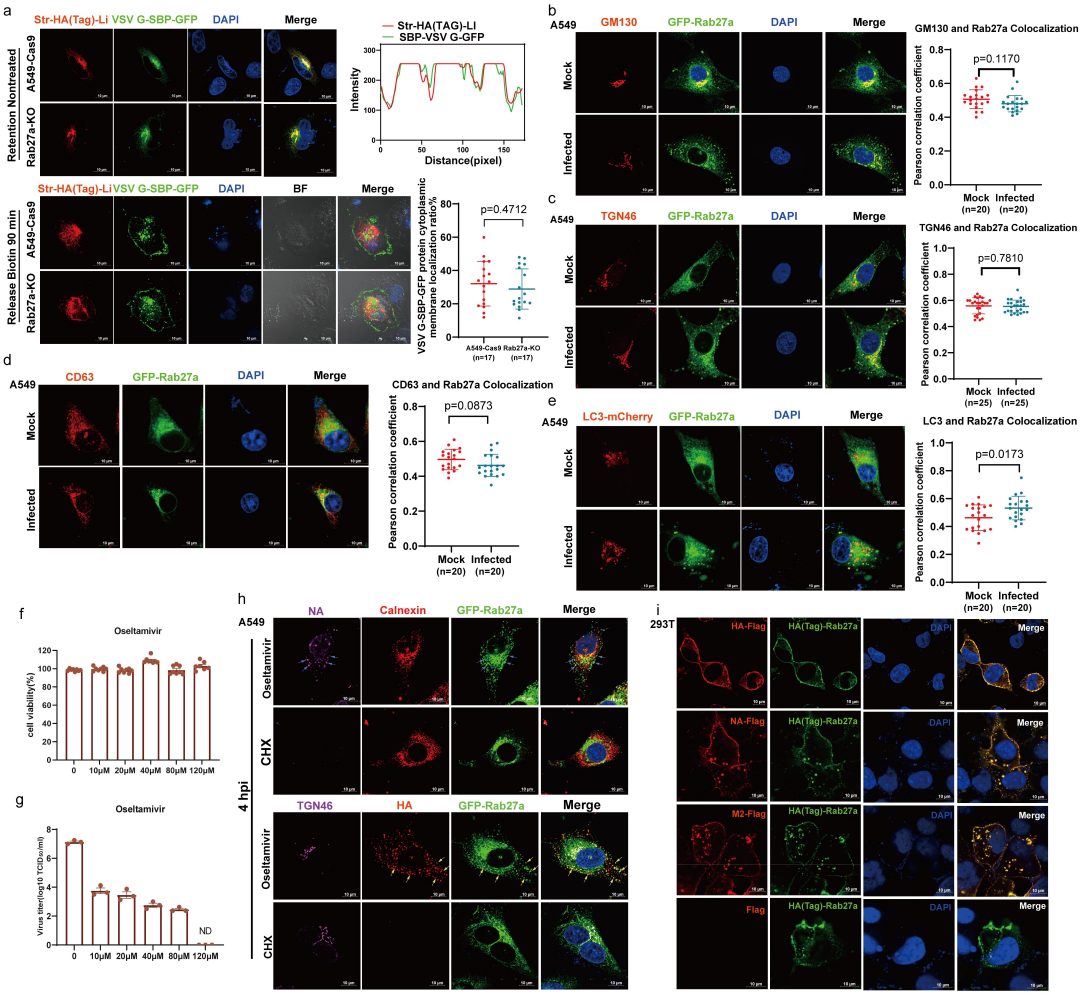
Supplementary Fig. 2. Rab27a regulates transport of HA and NA proteins to plasma membrane

a A549-Cas9, Rab27a-KO, and Rab27a-KO cells stably expressing Flag-Rab27a-WT, Flag-Rab27a-T23N, or Flag-Rab27a-Q78L were infected with HM virus (MOI, 10). At 6 hpi, cells were fixed, permeabilized, and stained with antibodies against HA (red) and counterstained with DAPI (blue). Scale bar, 10 μm. Representative images are shown from at least three independent experiments. **b, c** A549 cells were infected with VSV (MOI, 10). At 1-7 hpi, total (**b**) and cell surface (**c**) levels of VSV-G protein were quantified by flow cytometry. **d, e** A549 cells transfected with siNC or siRab27a (siRab27a-1, siRab27a-2) for 24 h were infected with VSV (MOI, 10). At 24 hpi, protein levels were detected by western blot in (**d**), and viral titers in supernatants were determined by $TCID_{50}$ assay on A549 cells (**e**). **f, g** A549 cells transfected with siNC or siRab6a (siRab6a-1, siRab6a-2) for 24 h were infected with VSV (MOI, 10). The cell surface (**f**) and Total (**g**) levels of VSV G protein were quantified by flow cytometry. Data are presented as mean ± SD from three independent experiments. Statistical significance was assessed using a two-tailed Student's t-test.



Supplementary Fig. 3. Rab27a promotes autophagy and M2 transport to plasma membrane

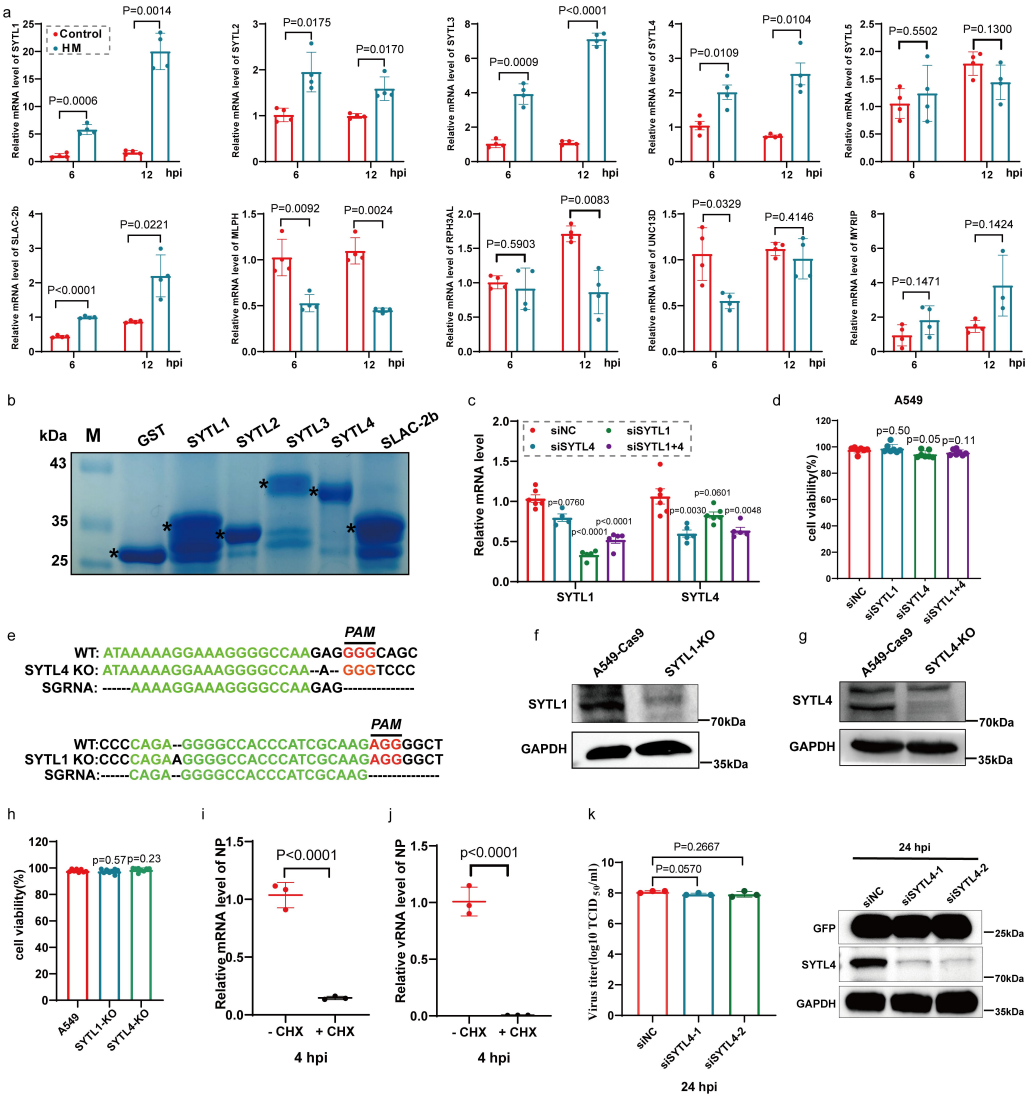
a A549 cells transfected HA-M2 or empty vector for 18 h, then treated with either MG132 (10 μM), CQ (100 μM), and DMSO for 12 h. **b** HEK293T cells were co-transfected with Rab27a-GFP and M2-Flag, NA-Flag, HA-Flag, or NP-Flag for 24 h. **c, d** HEK293T cells were co-transfected with Rab27a-Flag and M2-Flag or vector for 24 h, then treated with either CQ (100 μM), MG132 (10 μM), 3MA (25 mM), DMSO, or PBS for 12 h. **e** HEK293T cells were co-transfected with Rab27a and M2 or empty vector for 36 h. IP was performed using an anti-GFP antibody. Western densitometry analysis was conducted using ImageJ software. The indicated proteins were detected by western blot, with GAPDH serving as a loading control.



Supplementary Fig. 4. Rab27a regulates the transport of viral membrane proteins to the plasma membrane through Rab27a positive vesicles

a Rab27a-KO and A549-Cas9 cells were transfected with Str-HA(Tag)-Li_VSV G-SBP-GFP for 24 h, followed by the addition of biotin (400 μM) to the culture medium. At 0 and 90 min after biotin addition, cells were fixed, permeabilized, and stained with anti-HA (Tag) antibodies (red). Co-localization of VSV G with the ER or proportion of VSV G on the cell surface was analyzed using Fiji (n = 17 cells for Rab27a-KO and A549-Cas9 cells). **b-d** A549 cells stably expressing GFP-Rab27a were infected with HM virus (MOI, 10) or left uninfected. At 4 hpi, cells were fixed, permeabilized, and stained with antibodies against GM130, TGN46, or CD63 (all in red). The co-localization of GFP-Rab27a with different organelles was quantified by calculating the PCC using Coloc 2 in Fiji. GM130 (**b**, cis-Golgi, n = 20 cells in mock and infected groups); TGN46 (**c**, trans-Golgi, n = 28 cells in mock and infected

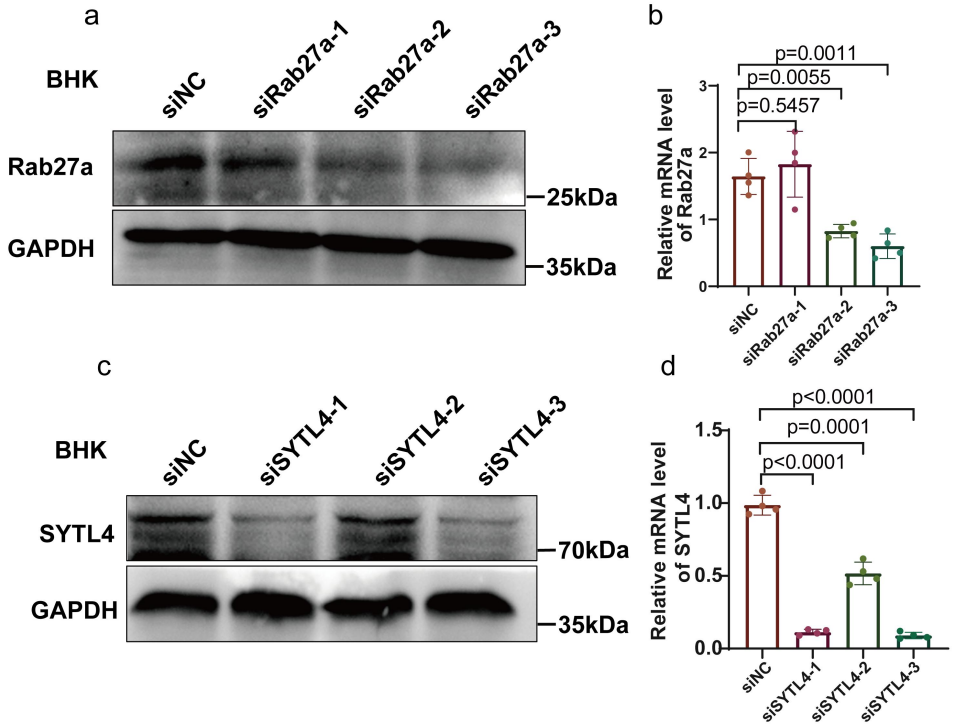
groups); CD63 (**d**, endosome, n = 20 cells in mock and infected groups). **e** A549 cells stably expressing GFP-Rab27a were transfected with LC3-mCherry for 24 h, then infected with HM virus (MOI, 10) or left uninfected. At 4 hpi, cells were fixed. The co-localization of GFP-Rab27a with LC3-mCherry was quantified by calculating the PCC using Coloc 2 in Fiji (n = 20 cells in mock and infected groups). **f** A549 cells were seeded into 96-well plates at 50% confluency and treated with 0–120 μM oseltamivir after cell attachment. Cell viability was assessed 12 h later using the CCK-8 assay. **g** A549 cells were treated with 0–120 μM oseltamivir, and the inhibitory effect on influenza virus replication was evaluated by the $TCID_{50}$ assay. **h** A549 cells stably expressing GFP-Rab27a were pretreated with CHX (100 μM) or DMSO for 4 h, and then infected with HM virus (MOI, 10). Cells were further treated with CHX (350 μM) or Oseltamivir (120 μM) and fixed at 4 hpi. These cells were stained with anti-NA, -Calnexin, -HA, and -TGN46 and DAPI. Blue arrows indicate colocalization of NA and GFP-Rab27a; yellow arrows indicate colocalization of HA and GFP-Rab27a. **i** HEK293T cells were co-transfected with HA-Flag, M2-Flag, or NA-Flag and HA (Tag) -Rab27a for 36 h. Then, cells were fixed, permeabilized, and stained with rabbit anti-Flag (red) and HA tag (green). Scale bar, 10 μm. Images shown are representative of at least three independent experiments. Statistical significance was determined using a two-tailed Student's t-test.



Supplementary Fig. 5. SYTL1 and SYTL4 are two Rab27a effectors involved in IAV membrane protein transport

a A549 cells were infected with or without HM virus (MOI, 10). At 6 and 12 hpi, samples were collected, and mRNA levels of Rab27a effectors were detected by qRT-PCR, with 18S rRNA used as the internal control. **b** Purified GST (lane 1) and GST-SHDs of SYTL1, SYTL2, SYTL3, SYTL4, and SLAC2b (lanes 2, 3, 4, 5, and 6) were detected using Coomassie blue staining. **c** A549 cells were transfected with siSYTL4, siSYTL1, or siNC for 24 h. Transcript levels of silenced genes were confirmed by qRT-PCR, with 18S rRNA as the internal control. **d** A549 cells were treated with siRNA against SYTL1, SYTL4, or nontargeting siRNA for 24 h, followed by measurement of cell viability using the CCK-8 assay. **e** Sequencing

results of SYTL1-KO, SYTL4-KO, and A549-Cas9 cells confirmed successful gene editing. **f, g** Protein levels of SYTL1 and SYTL4 in A549-Cas9, SYTL1-KO, and SYTL4-KO cells were detected by western blot. GAPDH served as the loading control. **h** SYTL4-KO, SYTL1-KO, and A549-Cas9 cells were seeded in a 96-well plate at equal cell numbers, with a density of ~50%. Cell viability was measured after 24 h using the CCK-8 assay. **i, j** A549-Cas9 cells were pretreated with CHX (100 μM) or DMSO for 4 h, then infected with HM virus (MOI, 10) and further treated with CHX (350 μM) or DMSO. Samples were collected at 4 hpi. mRNA and vRNA levels of the viral NP gene were detected by qRT-PCR, with viral RNA levels normalized to 18S rRNA. **k** A549 cells were transfected with siNC or siSYTL4 (siSYTL4-1, siSYTL4-2) for 24 h, then infected with VSV-GFP (MOI, 10). At 24 hpi, VSV protein levels were detected by western blot, and viral titers in supernatants were determined by $TCID_{50}$ assay on A549 cells. Data presentation: Data are presented as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed Student's t-test.



Supplementary Fig. 6. SYTL4 is a potential anti-influenza virus drug target

a, b BHK cells were transfected with siNC, siRab27a-1, siRab27a-2, siRab27a-3 for 24 h. Protein levels of Rab27a were detected by western blotting (**a**), and transcript levels were quantified by RT-qPCR (**b**). **c, d** BHK cells were transfected with siNC or siSYTL4-1, siSYTL4-2, siSYTL4-3 for 24 h. Protein levels of SYTL4 were detected by western blotting (**c**), and transcript levels were quantified by RT-qPCR (**d**). GAPDH was used as a loading control for western blotting, and mRNA levels were normalized to 18S rRNA for RT-qPCR analysis. Data are presented as means ± SD from three independent experiments. Statistical significance was determined using a two-tailed Student's t-test.