Microfilaments and Microtubules Alternately Coordinate the Multistep Endosomal Trafficking of Classical Swine Fever Virus

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ABSTRACT The cytoskeleton, as a ubiquitous structure in the cells, plays an important role in the processes of virus entry, replication, and survival. However, the action mechanism of the cytoskeleton in the invasion of pestivirus into host cells remains unclear. In this study, we systematically dissected the key roles of the main cytoskeleton components, namely microfilaments and microtubules, in the endocytosis of the porcine pestivirus classical swine fever virus (CSFV). We observed the dynamic changes of actin filaments in CSFV entry. Confocal microscopy showed that CSFV invasion induced the dissolution and aggregation of stress fibers, resulting in the formation of lamellipodia and filopodia. Chemical inhibitors and RNA interference were used to find that the dynamic changes of actin were caused by an EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42-cofilin signaling pathway, which regulates the microfilaments to help CSFV entry. Furthermore, colocalization of the microfilaments with clathrin and Rab5 (early endosome), as well as that of microtubules with Rab7 (late endosome) and Lamp1 (lysosome) revealed that microfilaments were activated and rearranged to help CSFV trafficking to the early endosome after endocytosis. Subsequently, recruitment of microtubules by CSFV also assisted membrane fusion of the virions from the late endosome to the lysosome with the help of a molecular motor, dynein. Unexpectedly, vimentin, which is an intermediate filament, had no effect on CSFV entry. Taken together, our findings comprehensively revealed the molecular mechanisms of cytoskeletal components that regulated CSFV endocytosis and facilitated further understanding of pestivirus entry, which would be conducive to exploration of antiviral molecules to control classical swine fever.

IMPORTANCE Endocytosis, an essential biological process mediating cellular internalization events, is often exploited by pathogens for their entry into target cells. Previously, we reported different mechanisms of CSFV endocytosis into the porcine epithelial cells (PK-15) and macrophages (3D4/21); however, the details of microfilaments/microtubules mediated virus migration within the host cells remained to be elucidated. In this study, we found that CSFV infection induced rearrangement of actin filaments regulated by cofilin through an EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42 pathway. Furthermore, we found that CSFV particles were trafficked along actin filaments in early and late endosomes, and through microtubules in lysosomes after entry. Here, we provide for the first time a comprehensive description of the cytoskeleton that facilitates the entry and the intracellular transport of a highly pathogenic swine virus. Results from this study will greatly contribute to the understanding of virus-induced early and complex changes in host cells that are important in CSFV pathogenesis.

KEYWORDS F-actin, endocytic pathway, microtubules, pestiviruses, vesicular trafficking

Pestiviruses cause economically important diseases among domestic ruminants and pigs, and they also infect a broad spectrum of wild even-toed ungulates (1, 2). There are three recognized species in the *Pestivirus* genus, classical swine fever virus

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(CSFV) (3), bovine viral diarrhea virus (4, 5), and border disease virus (6, 7). Classical swine fever (CSF), caused by CSFV, is an epidemic disease with high morbidity and high mortality that affects the pig industry worldwide, particularly in China (8, 9). At present, CSFV outbreaks still occur sporadically around the globe, including in countries that previously eradicated the disease (10–13). To explore novel and effective antiviral strategies to control the disease, it is essential to understand the molecular mechanisms of CSFV infection and pathogenicity, especially host-virus interactions.

The cytoskeleton is a cell's framework, composed of actin filaments, microtubules, and intermediate filaments, as defined based on filaments diameter and assembly patterns (14). They play important roles not only in the maintenance of cellular structure and morphology (15), but also in various cellular processes, such as cell movement (16, 17), material transport (18, 19), energy conversion (20, 21), information transmission (22, 23), and cell apoptosis (24). Several studies have shown that many viruses use the cytoskeleton of host cells to successfully complete their viral life cycle (25–27). Not surprisingly, many viruses interact with actin filaments and associated signaling pathways within the host cell because the actin cytoskeleton is a dynamic assembly of structures involved in many crucial cellular processes (28–30). Similarly, previous reports described that some members of the genera *Flavivirus* and *Hepacivirus* within the *Flaviviridae* family utilize host actin for efficient entry into the host cells (31, 32). However, the role of actin filaments during *Pestivirus* infection, specifically viral entry, has not been studied yet. Moreover, microtubules and the associated proteins also play essential roles in trafficking viral particles into host cells. It has been shown that disruption of the microtubule network affects the trafficking of West Nile virus structural proteins in infected cell (33, 34). Last, the intermediate fibers are the most stable and complex in structure among the three cytoskeletal fibers, and they mainly play a supporting role. The intermediate fibers are distributed around the nucleus, forming bundles and a net connected with the plasma membrane (35).

Recently, we showed that CSFV enters porcine kidney epithelial (PK-15) cells via clathrin-dependent endocytosis (36), whereas entry into porcine alveolar macrophages (3D4/21) is mediated by caveolin-dependent endocytosis (37). However, the molecular mechanisms of virus-containing vesicles trafficking through the cytoskeletal highway after endocytosis remain to be determined. In this study, we investigated the entry process of CSFV into PK-15 cells using a series of inhibitors and a small interfering RNA (siRNA) assay. The results showed that activation of the EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42-cofilin complex pathway induced rearrangement of F-actin that guided CSFV entry through clathrin-mediated endocytosis. Notably, we further confirmed the trafficking of the viral particles on the cytoskeleton highway by the transmission of endosomes, which elaborated the details of endosomal trafficking of CSFV after entry. These findings will provide insights for the identification of molecular targets for the development of antivirals that inhibit CSFV entry and subsequent viral replication.

RESULTS

CSFV infection induces actin rearrangement. For the normal development of follow-up experiments, cell viability upon exposure to each of the drugs and to siRNA were assessed using the Cell Counting Kit-8. As shown in Fig. 1, the concentrations of the drugs and siRNA used in this study are nontoxic. To determine whether CSFV infection induces F-actin rearrangements, we examined the expression of F-actin in PK-15 and 3D4/21 cells infected with CSFV (multiplicity of infection [MOI] = 10) at various time points by immunofluorescence assay using a laser confocal microscope. As shown in Fig. 2A, many F-actin stress fibers were evenly distributed in mock-infected cells. Interestingly, F-actin stress fibers became dissolved and accumulated at 10 min postinfection (mpi). At 60 mpi, stress fiber dissolution and accumulation increased with a larger number of F-actin stress fibers, in which lamellipodia and filopodia were observed. Consistent with F-actin expression changes observed in PK-15 cells, an increased number of lamellipodia and filopodia and accumulation of F-actin on the cell plasma membrane

FIG 1 Cell viability upon all the drugs was assessed using the Cell Counting Kit-8.

were detected at 10 mpi in 3D4/21 cells (Fig. 2B). To examine whether rearrangements of F-actin regulate CSFV infection, we further studied the effects of three F-actin inhibitors, latrunculin A (Lat A), cytochalasin D (Cyto D), and jasplakinolide (Jas), during CSFV infection in PK-15 cells. Cells were pretreated with nontoxic concentrations of each drug (Fig. 1) at 37°C for 1 h, followed by CSFV (MOI=10 or 1) inoculation at 4°C for 1 h, then shifted to 37°C for viral entry into the cells. Infected cell lysates were collected at 0 h postinfection (hpi) (binding), 1 hpi (entry), or 24 hpi (replication) to determine the viral RNA copy numbers by reverse transcription-quantitative PCR (RT-qPCR). The results showed that all three drugs significantly inhibited CSFV entry and replication in a dose-dependent manner but had no effect on viral binding (Fig. 2C to E). Viral RNA copies for entry and replication were reduced by 56.46% and 39.83% at 80 nM Jas, 56.49% and 61.5% at 8 nM Lat A, and 58.93% and 48.65% at 200 nM Cyto D compared to dimethyl sulfoxide (DMSO)-treated cells. The data suggested that inhibition of normal F-actin function by each drug blocks CSFV entry,

FIG 2 Role of the actin cytoskeleton in CSFV entry. (A) CSFV infection-induced F-actin rearrangement. PK-15 cells were infected with CSFV (multiplicity of infection [MOI] = 10). At 5, 10, and 60 min postinfection (mpi), cells were immunostained to detect (Continued on next page)

resulting in reduced viral replication. Moreover, we used confocal microscopy to confirm that Lat A, Cyto D, and Jas inhibit rearrangements induced by CSFV infection. To do this, cells were pretreated with each drug and infected with CSFV for 1 h. Based on the immunofluorescence assay coupled with confocal microscopy, F-actin rearrangements were clearly inhibited by all three inhibitors used in this study (Fig. 2F). Overall, F-actin is involved in CSFV entry and plays a critical role in the internalization of CSFV.

Modulation of cofilin activity affects CSFV entry. Cofilin is one of the essential proteins that can regulate the rearrangements of F-actin, and it plays an indispensable role in the process of stabilizing and depolymerizing F-actin (38). LIM domain kinase (LIMK) is the upstream kinase of cofilin; it can phosphorylate the third serine of cofilin, and it inhibits depolymerization of F-actin (39, 40). To investigate the role of cofilin during CSFV infection, we measured the expression of phosphorylated cofilin (p-cofilin) and cofilin in infected cells. Western blotting showed that p-cofilin expression decreased from 5 mpi to 90 mpi, recovered from 90 mpi to 120 mpi, and then decreased again at 150 mpi compared to that of mock-infected cells. In contrast, cofilin expression remained unchanged from 5 mpi to 30 mpi, decreased from 60 mpi to 120 mpi, and recovered at 150 mpi compared to that of mock-infected cells (Fig. 3A). The results indicate that there is a dynamic change on the expression of cofilin after virus infection that may regulate depolymerization and repolymerization of microfilaments. Next, to investigate whether cofilin is involved in various stages of CSFV infection, cells were treated with different concentrations of BMS-5 (Fig. 1), a specific inhibitor of upstream kinase LIMK1/2 of cofilin, and then infected with CSFV (MOI=10 or 1). RT-qPCR showed that BMS-5 significantly promoted entry and replication of CSFV in a dose-dependent manner, but had no effect on virus binding to cells (Fig. 3B). Subsequently, confocal microscope was used to observe the changes of p-cofilin from 30 to 150 mpi. As shown in Fig. 3C and D, in mock-infected cells, p-cofilin was mainly located in the nucleus. At 30 mpi, p-cofilin was dispersed to the cytoplasm, and at 150 mpi, p-cofilin gathered on the cell lamellipodia and filopodia (28). In addition, F-actin rearrangements were clearly inhibited by the BMS-5 used in this study (Fig. 2F). Taken together, the results suggested that cofilin is involved in regulation of the F-actin cytoskeleton and CSFV infection, and cellular localization of p-cofilin is closely associated with its function in regulating the F-actin cytoskeleton.

The Rho family is involved in CSFV entry. Rearrangements of the actin cytoskeleton are generally regulated by Rho GTPase signaling pathways (41). RhoA, Rac1, and Cdc42 are the best characterized Rho GTPases and regulate many actin-driven processes. RhoA induces formation of stress fibers, while activation of Rac1 and Cdc42 induces polymerization of actin and formation of a network of actin filaments underlying the plasma membrane (42, 43). Thus, Rho GTPases are potential candidates for mediating signaling between actin and endocytic trafficking (44) and may be involved in the endocytic entry and trafficking of CSFV. First, cells were pretreated with different concentrations of three Rho inhibitors (Fig. 1), Rhosin, NSC23766, and ML141, followed by CSFV infection (MOI=10 or 1). At 0 hpi (binding), 1 hpi (entry), or 24 hpi (replication), infected cells were lysed to determine the viral RNA copy numbers by RT-qPCR. As expected, the results showed that Rhosin, NSC23766, and ML141 significantly decreased the entry and replication of CSFV in a dose-dependent manner but had no effect on virus binding to cells (Fig. 4A to C). Second, to further determine that Rho GTPases promote CSFV infection, we used the siRNA duplex to knockdown endogenous RhoA, Rac1, or

FIG 2 Legend (Continued)

endogenous actin using fluorescein isothiocyanate (FITC)-phalloidin [microfilament green fluorescent probe dye]. Bars, 10 μ m. (B) 3D4/21 cells were infected with CSFV (MOI=10). At 10 mpi, cells were fixed with 4% paraformaldehyde (PFA) and stained with FITC-phalloidin (green), then observed by confocal microscopy. Bars, 10 μ m. (C, D, and E) Latrunculin A (Lat A), cytochalasin D (Cyto D), and jasplakinolide (Jas) inhibited CSFV entry and replication. PK-15 cells were pretreated with increasing concentrations of drugs targeting F-actin, namely latrunculin A (Lat A), cytochalasin D (Cyto D), and jasplakinolide (Jas), at 37°C for 1 h, inoculated with CSFV (MOI=10 or 1) at 4°C for 1 h, and then shifted to 37°C. At 0 (binding), 1 (entry), or 24 (replication) h postinfection (hpi), the cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ standard deviation (SD) of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (F) PK-15 cells were pretreated with the indicated inhibitors and infected with CSFV (MOI=10) for 1 hpi. Then, cells were stained with FITC-phalloidin (green) and observed by confocal microscopy. Bars, 10 μ m.

FIG 3 Modulation of cofilin activity affects CSFV entry. (A) Cells were incubated with CSFV (MOI = 10) at 4°C for 1 h, then shifted to 37°C. At the indicated time points, protein expression of phosphorylated cofilin (p-cofilin) and cofilin were measured by Western blotting. (B) BMS-5 inhibited CSFV entry and replication. Cells were pretreated with different concentrations of BMS-5 at 37°C for 1 h, inoculated with CSFV (MOI = 10 or 1) at 4°C for 1 h, and then shifted to 37°C. At 0 (binding), 1 (entry), or 24 (replication) hpi, the cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) CSFV infection induces p-cofilin position change. Cells were fixed at the indicated time points and immunostained to detect endogenous actin and p-cofilin using FITC-phalloidin and anti-p-cofilin antibody. Bars, 10 μ m. (D) Cells were fixed at the indicated time points and immunostained to detect CSFV and p-cofilin using mouse anti-E2 and rabbit anti-p-cofilin antibodies. Bars, 10 μ m.

Cdc42 (Fig. 4D). RT-qPCR showed that siRNA specific for each gene inhibited CSFV entry at 1 hpi and replication at 24 hpi, respectively, suggesting that CSFV endocytosis inhibition caused a reduction in viral replication (Fig. 4E). The results showed that the protein expression of RhoA, Rac1, Cdc42, and CSFV Npro decreased significantly (Fig. 4E), as detected by Western blotting. Third, to investigate whether ROCK of the RhoA downstream (45) and PAK of the Rac1 and Cdc42 downstream (46) were activated in the early stage of CSFV infection, we first detected the expression of ROCK1 and p-PAK1 after infection at different time points. Western blotting showed that the expression levels of ROCK1 and p-PAK1

FIG 4 The Rho family is involved in CSFV infection. (A, B, and C) Rhosin, NSC23766, and ML141 inhibited CSFV entry and replication. Cells were pretreated with different concentrations of Rhosin, NSC23766, and ML141, respectively, at 37°C for 1 h, inoculated with CSFV (MOI=10 or 1) 4°C for 1 h, then shifted to 37°C. At 0 (binding), 1 (entry), or 24 (replication) hpi, the cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D and E) The siRhoA, siRac1, siCdc42, or siCtrl-transfected cells were inoculated with CSFV (MOI=10 or 1). At 1 and 24 hpi, the cell cultures were harvested for RT-qPCR or Western blotting. (F and G) Cells were incubated with CSFV (MOI=10) at 4°C for 1 h and then shifted to 37°C. Expression of ROCK1, p-PAK1, and PAK1 were measured by Western blotting. (H and I) Y-27632 and IPA-3 inhibited CSFV entry and replication. Cells were pretreated with different concentrations of Y-27632 and IPA-3, respectively, as described above. The cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

increased until 150 mpi (Fig. 4F and G). It is suggested that CSFV significantly activated RhoA/ROCK and Rac1/Cdc42/PAK signaling in the early stage of infection. Alternatively, we used two drugs (Fig. 1), Y-27632 (inhibitor of ROCK1) and IPA-3 (inhibitor of PAK), to further support the data above. RT-qPCR showed that Y-27632 and IPA-3 significantly decreased the entry and replication of CSFV in a dose-dependent manner but had no effect on virus binding to cells (Fig. 4H and I). In addition, confocal microscopy was performed to prove further the regulating effect of RhoA/ROCK and Rac1/Cdc42/PAK pathways on F-actin. Drug treatment inhibited the polymerization of F-actin induced by CSFV and the formation of lamellipodia and filopodia on the cell membrane (Fig. 2F). These results indicated that cells infected with CSFV significantly activated RhoA/ROCK and Rac1/Cdc42/PAK pathways in the early stage of infection, which helps CSFV endocytosis by regulating the dynamic changes of the F-actin.

PI3K/Akt and MEK/ERK1/2-MAPK pathways are involved in CSFV entry. In the regulation of the actin cytoskeleton signaling pathway, MEK/ERK regulates the activity of ROCK1, whereas PI3K regulates the activity of PAK1. Finally, both of them regulate the rearrangement of the actin cytoskeleton (29, 31). First, to prove that these signaling pathways are involved in Rho GTPase regulation, cells were pretreated with increasing concentrations of U0126 (a potent, non-ATP competitive and selective MEK1/2 inhibitor) or OSU-03012 (an inhibitor of the PI3K/PDK1/AKT signaling pathway) and infected with CSFV (MOI = 10) for 1 h at 37°C, and the levels of ROCK1 and p-PAK1 were examined by using Western blotting. The results showed that the protein expression of ROCK1 and p-PAK1 decreased in a dose-dependent manner (Fig. 5A and B). Second, to investigate whether the MEK/ERK and PI3K pathways were activated during the process of CSFV entry, the expression levels of p-PI3K and p-MAPK were measured in cells infected with CSFV at the indicated time points by Western blotting. The results showed that the levels of p-PI3K and p-MAPK increased rapidly since 5 mpi (Fig. 5C and D), suggesting that CSFV significantly activates PI3K/Akt and MEK/ERK1/2 pathways in the early stage of infection. Next, we used the inhibitors to investigate the effect of these two pathways on CSFV infection. Cells were pretreated with OSU-03012 or U0126 and subsequently infected with CSFV (MOI = 10 or 1). At 0 hpi (binding), 1 hpi (entry), or 24 hpi (replication), infected cells were lysed to determine the viral RNA copy numbers by RT-qPCR. The results showed that OSU-03012 and U0126 significantly inhibited the entry and replication of CSFV in a dose-dependent manner but had no effect on the binding of CSFV (Fig. 5E and F). Furthermore, cells were pretreated with LY294002, a PI3K inhibitor, and infected with CSFV (MOI = 10). At 0 hpi (binding), 1 hpi (entry), infected cells were lysed to determine the viral RNA copy numbers by RT-qPCR. The results showed that LY294002 significantly inhibited viral entry in a dose-dependent manner but had no effect on the binding of CSFV (Fig. 5G). Thus, we directly assessed the role of PI3K during CSFV entry by using a siRNA assay. The Western blotting showed that the protein levels of PI3K and CSFV-Npro decreased significantly (Fig. 5H). To investigate whether these two pathways were related to the rearrangements of F-actin, cells were pretreated with OSU-03012 or U0126, followed by CSFV (MOI = 10) infection, and analyzed with a confocal microscope. The images showed that OSU-03012 and U0126 inhibited the dynamic changes of F-actin induced by CSFV (Fig. 2F). Overall, these results indicated that infected cells significantly activated PI3K/Akt and MEK/ERK1/2 signal pathways in the early stage, which regulated the dynamic changes of F-actin during the invasion of CSFV.

CSFV entry is dependent on EGFR. It is well known that there are two main signaling pathways downstream of EGFR, the PI3K/Akt and MEK/ERK1/2-MAPK pathways (47). In addition, EGFR is a coreceptor for many viruses that help to invade the host cells (48, 49). Once activated, EGFR can regulate the rearrangements of F-actin in the host cells following clathrin-mediated viral endocytosis (50). Therefore, we speculate that EGFR is also related to CSFV entry into PK-15 cells. To determine if EGFR can affect the activities of PI3K/Akt and MEK/ERK1/2-MAPK pathways, expression levels of p-PI3K, p-MAPK, and p-EGFR were measured in cells pretreated with increasing concentrations of AG-1478 and infected with CSFV (MOI = 10) for 1 h at 37°C by Western blotting. The results showed that the protein levels of p-PI3K, p-MAPK, and p-EGFR decreased in a

FIG 5 PI3K/Akt and MEK/ERK1/2-MAPK signal pathways are involved in CSFV entry. (A and B) Cells were pretreated with different concentrations of OSU-03012 and U0126, respectively, for 1 h and incubated with CSFV (MOI=10) at 4°C for 1 h then shifted to 37°C for 1 h. Expression of ROCK1, p-PAK, and PAK was measured by Western blotting. (C and D) Cells were incubated with CSFV (MOI=10) at 4°C for 1 h, then shifted to 37°C. Expression of p-PI3K, PI3K, p-MAPK, and MAPK were measured by Western blotting. (E, F, and G) The inhibitors of PI3K or MAPK signal pathway impaired CSFV entry and replication. Cells were pretreated with different concentrations of LY294002, OSU-03012, or U0126 as described above. The cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (H) Knockdown of PI3K inhibited CSFV infection. The siPI3K or siCtrl-transfected cells were infected with CSFV (MOI=1) and harvested at 24 hpi for Western blotting using the indicated antibodies.

dose-dependent manner (Fig. 6A), indicating that the activity of PI3K or MAPK pathways was inhibited by blocking phosphorylation of EGFR. To investigate whether EGFR was activated during the early stage of CSFV infection, p-EGFR levels were measured in cells infected with CSFV (MOI=10) at different time points (5 to 150 min) by Western blotting. The results showed that the level of p-EGFR increased from 10 to 60 mpi and decreased to normal level by 150 mpi (Fig. 6B), indicating that CSFV entry significantly activated EGFR. Next, cells were pretreated for 1 h with different concentrations of epidermal growth factor (EGF, an activator for the phosphorylation of EGFR) or AG-1478 (EGFR-specific inhibitor) (Fig. 1), followed by inoculation with CSFV for 1 h at 4°C, and then shifted to 37°C for 0 (binding), 1 (entry), or 24 (replication) hpi. The amount of virus at binding, internalization, or replication steps was determined by quantifying viral RNA using RT-qPCR. The results showed that EGF significantly promoted the entry and replication of CSFV in a dose-dependent manner, but did not promote binding,

FIG 6 CSFV entry depends on EGFR. (A) AG-1478 inhibited PI3K and MAPK activation. Cells were pretreated with increasing concentrations of AG-1478 at 37°C for 1 h, inoculated with CSFV (MOI = 10) at 4°C for 1 h, and then shifted to 37°C for 1 h. Levels of p-PI3K, PI3K, p-MAPK, MAPK, p-EGFR, and EGFR were measured by Western blotting. (B) EGFR was activated in the early

(Continued on next page)

suggesting that EGF activated EGFR and then enhanced CSFV endocytosis (Fig. 6C). On the contrary, AG-1478 significantly inhibited the entry and replication of CSFV in a dose-dependent manner (Fig. 6D). Western blotting further confirmed that the protein level of CSFV Npro decreased gradually along with the increasing concentrations of AG-1478 (Fig. 6E). Moreover, cells were infected with CSFV (MOI=10) at 37°C (set as 0 h). AG-1478 was added to the cells at different time intervals, as follows: -1 to 0, 0 to 0.5, 0.5 to 1, 1 to 1.5, and 1.5 to 2 h. At 2 hpi, infected cells were lysed to determine the viral RNA copy numbers by RT-qPCR. The results showed that the viral RNA copy numbers were markedly decreased at -1 to 1.5 h (Fig. 6F), suggesting that EGFR helps CSFV entry at the early stage of the viral life cycle. In addition, cells pretreated with AG-1478 and infected with CSFV (MOI=10) for 1 h were observed using confocal microscopy. As described above (Fig. 2F), AG-1478 inhibited depolymerization and polymerization of F-actin stress fibers induced by CSFV and the production of lamellipodia and filopodia. Subsequently, to examine whether EGFR regulated CSFV entry using confocal microscopy, cells were inoculated with CSFV (MOI=10) at 4°C for 1 h to allow attachment and then shifted to 37°C for 10, 60, and 120 min for entry. The localization of virions with p-EGFR was obvious at indicated time points (Fig. 6G). We concluded that CSFV entry into PK-15 cells is dependent on EGFR. At last, we used the siRNA duplex to knockdown the endogenous EGFR, and used RT-qPCR and WB to detect the changes of viral RNA and Npro protein expression at 1 and 24 hpi. The results showed that viral RNA replication was significantly downregulated along with EGFR knockdown at 1 hpi (Fig. 6H). Moreover, the viral CSFV RNA and Npro protein expression was significantly reduced at 24 hpi (Fig. 6I). This result further confirmed the important role of EGFR in CSFV infection of PK-15 cells. Overall, these results suggested that CSFV significantly activates EGFR in the early stage, and activated EGFR can affect the invasion of CSFV by regulating the dynamic changes of F-actin.

Actin filaments regulate CSFV endocytosis. The above results demonstrated that the EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42-cofilin pathway activated F-actin rearrangement and assisted CSFV in entering PK-15 cells. Our previous studies confirmed that CSFV entry into PK-15 cells requires clathrin and early endosomes during the endocytosis (36). We hypothesized that actin filaments were involved in this trafficking from clathrin to early endosomes. To this end, PK-15 cells were infected with CSFV (MOI=10), fixed at different time points (15 mpi, 30 mpi, and 60 mpi), and stained with specific antibodies for confocal microscopy. We found that 15 min after the virus entered the cell, F-actin, clathrin, and virions were colocalized. The superimposed fluorescent spots formed a hemispherical shape, indicating that the virus was entering the cell from the outside. At 30 mpi, the superimposed fluorescence signals were more intense, and the numbers of colocalization points were significantly increased. However, at 60 mpi, the colocalized fluorescent spots began to decrease (Fig. 7A), indicating that most of the virus particles had entered into the cells and were then driven by clathrin-actin filaments to the next trans-

FIG 6 Legend (Continued)

stage of CSFV infection. PK-15 cells were incubated with CSFV (MOI=10) at 4°C for 1 h, then shifted to 37°C. At the indicated time points, the cell cultures were harvested, and protein expression of p-EGFR and EGFR was measured by Western blotting. (C) The activation of EGFR promotes CSFV entry and replication. PK-15 cells were pretreated with increasing concentrations of EGF at 37°C for 1 h, inoculated with CSFV (MOI=10 or 1) at 4°C for 1 h, and then shifted to 37°C for 0 (binding), 1 (entry), or 24 (replication) hpi. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) AG-1478 inhibited CSFV entry and replication. Cells were pretreated with different concentrations of AG-1478 at 37°C for 1 h, inoculated with CSFV (MOI=10 or 1) at 4°C for 1 h, and then shifted to 37°C for 0 (binding), 1 (entry), or 24 (replication) hpi. Viral RNA copies were determined by RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E) Cells were pretreated with increasing concentrations of AG-1478 at 37°C for 1 h, inoculated with CSFV (MOI=1) at 4°C for 1 h, and then shifted to 37°C for 24 hpi. Expression of Npro and viral RNA copies were measured by Western blotting and RT-qPCR, respectively. (F) AG-1478 inhibited CSFV entry at the early stage. Cells were pretreated with AG-1478 for 1 h at 37°C. Alternatively, PK-15 cells were incubated with CSFV (MOI=10) at 4°C for 1 h, and unbound viruses were removed. Cells were shifted to 37°C, and AG-1478 was added at 0 to 0.5 hpi, 0.5 to 1 hpi, 1 to 1.5 hpi, and 1.5 to 2 hpi. Viral RNA copies were determined by RT-qPCR. (G) CSFV colocalized with p-EGFR. Cells infected with CSFV (MOI=10) were kept at 4°C for 1 hpi, then shifted to 37°C. At different time intervals, cells were then immunostained to detect endogenous EGFR (green) and viral particles (red). Bars, 10 μ m. (H and I) The siEGFR- or siCtrl-transfected cells were inoculated with CSFV (MOI=10 or 1). At 1 and 24 hpi, the cell cultures were harvested for RT-qPCR and Western blotting.

FIG 7 CSFV entry is dependent on actin filaments. (A) Actin is involved in clathrin-dependent endocytosis. Cells were infected with CSFV (MOI=10) and fixed at 15, 30, or 60 mpi. Cells were then immunostained to detect endogenous clathrin (purple), actin filaments (green), or viral particles (red). Bars, 10 μm. (B) Cells were infected with CSFV (MOI=10) and fixed at 0.5, 1.5, or 4 hpi. Cells were then immunostained to detect endogenous Rab5 (purple), actin filaments (green), or viral particles (red). Bars, 10 μm.

port stop, the early endosomes. Therefore, as shown in Fig. 7B, the images showed Rab5, CSFV particles, and F-actin with lamellipodia were colocalized at 30 mpi; at 90 mpi, there was clear colocalization among Rab5 and virions and between F-actin and dissolved stress fibers. At 4 hpi, reduced colocalization was observed, suggesting that transport of early endosomes did not need the help of F-actin at a later time. Overall, these results suggested that CSFV particles “run” along the clathrin on the microfilament highway to the early endosome.

Microtubules regulate CSFV trafficking from late endosomes to lysosomes. To investigate whether microtubules act as the next “baton” and play a significant role

in endocytosis of CSFV, PK-15 cells were infected with CSFV, and nocodazole (a microtubule inhibitor) was added to cells at different time points. The viral RNA in cells was detected by RT-qPCR. The results showed that adding nocodazole 1 h in advance and 3 h after virus infection could significantly inhibit the invasion of CSFV. In contrast, the inhibitory effect was not apparent when nocodazole was added within 1 h of virus infection (Fig. 8A). It is speculated that microtubules play a crucial role in the late stage of CSFV entry. Furthermore, to investigate whether late endosomes and lysosomes were transported on the microtubule highway post-endocytosis, cells were infected with CSFV (MOI = 10) and fixed at different time points (1, 2, and 4 hpi). The colocalization of tubulin, Rab7, and virions and that of tubulin, Lamp1, and virions were observed by confocal microscope (Fig. 8B and D). The results showed that among tubulin, Rab7, and CSFV, and among tubulin, Lamp1, and CSFV, there were apparent colocalization. The colocalization coefficients were expressed as Pearson's correlation coefficient, measured for individual cells. As shown in Fig. 8C and E, Pearson's correlation coefficient is significant (>0.4) during CSFV endocytosis. However, Rab10 did not colocalize with tubulin and CSFV as a negative control (Fig. 8F and G). These results suggested that microtubules regulate Rab7-mediated endosome and Lamp1-mediated lysosome trafficking in the early stage of CSFV infection.

CSFV uses dynein to infect cells efficiently. Microtubule motors are used for the bidirectional transport of cargo. Minus-end motors (dynein) transport cargo toward the cell interior, whereas plus-end motors (kinesin) move cargo toward the cell periphery (51, 52). It is not known whether microtubules or microtubule motors are required for reovirus entry. Here, we carried out a series of experiments to determine the role of dynein in the process of CSFV infection of PK-15 cells. First, we pre-treated PK-15 cells with ciliobrevin D ($20\mu\text{M}$), a specific inhibitor of dynein, and infected CSFV. RT-qPCR showed that viral RNA copies were reduced by 38.63% at 1 hpi, 56.74% at 2 hpi, and 37.99% at 6 hpi after treatment compared to DMSO-treated cells (Fig. 9A). Next, the dynein knockdown cells were infected with CSFV, and the viral RNA level was measured. As shown in Fig. 9B, viral RNA copies were reduced by 21.24% at 1 hpi, 62.16% at 2 hpi, and 71.46% at 6 hpi compared to those in control cells. The results were consistent with those after treatment with an inhibitor, which further confirmed that dynein is needed for CSFV entry. Finally, cells were inoculated with purified CSFV virions (MOI = 10) at 4°C for 1 h to allow attachment and shifted to 37°C for 1 h, 3 h, and 6 h before fixation. The localization of virions with dynein and microtubules was analyzed by confocal microscopy. Virions, dynein, and microtubules showed significant colocalization at 1, 3, and 6 hpi (Fig. 9C). We concluded that CSFV entry into PK-15 cells is dependent on the microtubule-based motor protein dynein, which helps to transport virus-carrying vesicles on microtubules to facilitate virus trafficking.

Vimentin is not involved in CSFV entry. Vimentin is one of the critical proteins in the intermediate filaments. Together with microfilaments and microtubules, vimentin constitutes the cytoskeleton system (53). Its primary function is to maintain the cell morphology and stabilize the cytoskeleton. According to the previous article, we know that microfilaments and microtubules play an essential role in the infection of PK-15 cells by CSFV. What role does the intermediate filament, which is one of the cytoskeleton components, play in the process of CSFV infection? To this end, we used CSFV (MOI = 10) to infect PK-15 cells, and the localization of virions with vimentin was analyzed by confocal microscopy. The results showed that vimentin did not colocalize with virions (Fig. 10A). Furthermore, RT-qPCR and Western blotting showed that viral RNA copies were not reduced compared to those in control cells at 1, 2, and 6 hpi after knockdown of vimentin (Fig. 10B). Overall, the results showed that CSFV entry did not require vimentin.

DISCUSSION

To successfully infect the host cells, CSFV must be transported to the lysosome after

entering the cell for membrane fusion, uncoating, and then replicating viral mRNA in the cytoplasm. Our previous studies have shown that CSFV particles were trafficked from the early and late endosome to the lysosome after entering the host cells (36). However, little was known until now about the trafficking of viral particles between the endosomes. In this study, we revealed the transmission pattern of virus particles between the endosome and lysosome with the regulation of actin filaments and microtubules (Fig. 11).

The cytoskeleton plays an essential role not only in maintaining cell morphology, bearing external forces, and maintaining the order of internal cell structure, but also in important life activities such as cell movement and material transport. Viruses usually promote their replication, transportation, assembly, and release by regulating the cytoskeleton (54, 55). In particular, in the entry process, cytoskeleton remodeling often promotes efficient entry of viruses into host cells, including that of HIV (27), influenza virus (26), and herpes simplex virus (56). Considering that virus entry is one of the essential steps in the cycle life of a virus, it is necessary to study cytoskeleton regulation in virus entry. Here, the relationship between CSFV endocytosis and cytoskeleton regulation of host cells will be studied to explore the molecular mechanism of cytoskeleton regulation and the influence on virus entry to enrich in-depth understanding of CSFV etiology. On the other hand, since there are few drugs to effectively control CSFV in clinical settings, the present study will further explore the role of the cytoskeleton in the process of CSFV infection and provide a scientific basis for the discovery of new antivirals based on cytoskeleton regulation.

As an essential regulatory protein of the cytoskeleton depolymerization factor family, cofilin is widely involved in cell migration (57). Also, it is an actin-binding protein that exists in eukaryotic cells and depolymerizes F-actin in cells (38). Its activity is to regulate the rearrangements of F-actin through phosphorylation, dephosphorylation, inositol phosphate, and pH change (58). Recent studies have shown that cofilin is also involved in the entry of bacteria (59) or the regulation of cytoskeleton caused by viruses (60–62). Our study found that the invasion of CSFV caused rearrangement of F-actin under the cell membrane (Fig. 2A), so we suspected that this rearrangement was regulated by cofilin. The results confirmed that the phosphorylation level of cofilin changed significantly after viral infection (Fig. 3A), consistent with previous reports. With the continuous use of chemical inhibitors and RNA interference assay, we gradually revealed an obvious signal pathway (EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42-cofilin) that regulated the phosphorylation of cofilin, which leads to the rearrangement of F-actin following by the entry of CSFV into host cells. Although this signal pathway has been reported in HSV1 infection (29), this is the first report in the study of porcine virus entry. It is a useful tool for elucidating the detailed mechanism of a vital cellular pathway.

In our previous work, we found that clathrin-mediated endocytosis was the main route for CSFV to infect PK-15 cells. After entering the cell, it was transported through the early endosome, late endosome, and lysosome (36). The colocalization of clathrin and F-actin indicated that the clathrin-coated vesicles carried CSFV to enter the cyto-

FIG 8 Legend (Continued)

(MOI=10) at 4°C for 1 h, unbound viruses were removed, cells were shifted to 37°C, and nocodazole was added at 0 to 1 hpi, 1 to 2 hpi, 1 to 4 hpi, and throughout, respectively. Viral RNA copies were determined by RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Cells were infected with CSFV (MOI=10) for 1, 2, and 4 h at 37°C, fixed, and then stained with rabbit anti-Rab7 antibody (purple), mouse anti-E2 antibody (green), and dye of microtubules (red), and observed by confocal microscopy. Bars, 10 μ m. (C) The colocalization analysis of tubulin, Rab7, and CSFV was expressed with Pearson's correlation coefficient. (D) The treated cells described above were stained with rabbit anti-Lamp1 antibody (purple), mouse anti-E2 antibody (green), and dye of microtubules (red), and observed by confocal microscopy. Bars, 10 μ m. (E) The colocalization analysis of tubulin, Lamp1, and CSFV was expressed with Pearson's correlation coefficient. (F) Cells were infected with CSFV and fixed at 1 or 4 hpi. Then, cells were probed with rabbit anti-Rab10 (purple), mouse anti-E2 antibody (green), and dye of microtubules (red), and observed by confocal microscopy. Bars, 10 μ m. (G) The colocalization analysis of tubulin, Rab10, and CSFV was expressed with Pearson's correlation coefficient.

FIG 9 Dynein is involved in CSFV entry. (A) Ciliobrevin D inhibited CSFV entry. PK-15 cells were pretreated with ciliobrevin D (μM) and dimethyl sulfoxide (DMSO) at 37°C for 1 h and inoculated with CSFV ($\text{MOI}=10$) at 4°C for 1 h and then shifted to 37°C for endocytosis. At 0, 1, 2, and 6 hpi, cell cultures were harvested for RT-qPCR. These data are presented as the mean $\pm$ SD of data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) The siDynein- or siCtrl-transfected cells were infected with CSFV ($\text{MOI}=10$) and harvested at 1, 2, and 6 hpi for RT-qPCR or Western blotting. (C) Cells were infected with CSFV ($\text{MOI}=10$), fixed at 1, 3, and 6 hpi, and then stained with rabbit anti-DHC antibody (purple), mouse anti-E2 antibody (green), and dye of microtubules (red), and observed by confocal microscopy. Bars, $10\mu\text{m}$.

plasm due to the cell membrane depression and then transmitted it on the microfilament highway. The colocalization of Rab5 and F-actin was further demonstrated by confocal microscopy, indicating that the early endosome took over the “baton” and continued to carry the virus along the microfilaments. However, the colocalization of F-actin with Rab5 and viral particles decreased at 4 hpi, suggesting that there is

FIG 10 CSFV entry does not need vimentin. (A) CSFV virions did not colocalize with vimentin. Cells were infected with CSFV (MOI=10) and fixed at 1, 2, and 4 hpi, and then stained with rabbit anti-vimentin antibody (green) and mouse anti-E2 antibody (red) and observed by confocal microscopy. Bars, 10 μ m. (B) Knockdown of vimentin did not affect CSFV entry. The vimentin siRNA-or siCtrl-transfected cells were infected with CSFV (MOI=10) and harvested at 1 hpi for RT-qPCR or Western blotting.

FIG 11 Schematic model depicting cytoskeleton network coordinates the multistep endosomal trafficking of CSFV after endocytosis in PK-15 cells. At the early stage of infection, CSFV stimulates the phosphorylation of cofilin through the EGFR-PI3K/MAPK-RhoA/Rac1/Cdc42 signaling pathway, followed by the rearrangement of F-actin. CSFV entry into PK-15 cells through clathrin-mediated endocytosis with the help of F-actin. It is then trafficked through the early endosomes to the late endosomes along the actin. CSFV is continuously trafficked through the late endosomes to the lysosomes along the microtubules, which require a molecular motor, dynein.

another highway to facilitate the trafficking of the virus during the transmission of the early endosome to late endosome (Fig. 7). Vesicle trafficking at the cell cortex might be actin-dependent before transfer of the vesicle to microtubules to complete the journey to the perinuclear region (63). Early studies have shown that microtubules regulate virus infection (64). As pathogenic cargos, viruses require microtubules for transport to and from their intracellular sites of replication (65). Nocodazole disrupts microtubules and inhibits vesicular trafficking, including transport between early and late endosomes. Our previous study showed that treatment of Japanese encephalitis virus (JEV)-infected cells with nocodazole significantly decreased the viral RNA copies, indicating that microtubules play an essential role in JEV productive infection by mediating trafficking of JEV-containing endosomes, which is in agreement with a previous study (66). Since JEV and CSFV both belong to the *Flaviviridae* family, we speculated microtubules might play a critical role during the infection of CSFV. As expected, nocodazole expectedly significantly decreased the early stage of CSFV infection, suggesting that microtubules played an important role. Confocal microscopy showed that the virus and microtubule were colocalized with Rab7 and Lamp1, respectively, indicating that the late endosome trafficked virus along microtubule to the lysosome (Fig. 8). Therefore, we confirmed that CSFV was transmitted alternately along the two highways of microfilaments and microtubules.

It is worth noting that we have described for the first time the role of molecular motors involved in the life cycle of classical swine fever. Dynein is a microtubule-based molecular motor that exclusively provides the driving force for fast retrograde transport, indispensable for many viruses to transport toward the cell nucleus (67–70). Here, we observed that dynein colocalized with CSFV and microtubules (Fig. 9), suggesting

TABLE 1 Antibodies used in this study

Antibody	Name ^a	Supplier	Catalog no.
Cofilin	Cofilin (D3F9) XP rabbit MAb	CST	5175
p-Cofilin	Phospho-cofilin (Ser3) (77G2) rabbit MAb	CST	3313
RhoA	RhoA (67B9) rabbit MAb	CST	2117
Rac1/Cdc42	Rac1/Cdc42 antibody	CST	4651
ROCK1	ROCK1 (C8F7) rabbit MAb	CST	4035
PAK1	PAK1 antibody	CST	2602
p-PAK1	Phospho-PAK1 (Ser199/204)/PAK2 (Ser192/197) antibody	CST	2605
MAPK	p44/42 MAPK (Erk1/2) (137F5) rabbit MAb	CST	4695
p-MAPK	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP	CST	4370
PI3K	PI3 kinase p85 (19H8) rabbit MAb	CST	4257
p-PI3K	Phospho-PI3K p85 alpha (Tyr607) polyclonal antibody	Invitrogen	PA5-38905
EGFR	EGF receptor (D38B1) XP rabbit MAb	CST	4267
p-EGFR	Phospho-EGFR (Tyr1068) polyclonal antibody	Invitrogen	PA5-17848
Clathrin	Clathrin heavy chain (D3C6) XP rabbit MAb	CST	4796
Rab5	Rab5 (C8B1) rabbit MAb	CST	3547
Rab7	Rab7 (D95F2) XP rabbit MAb	CST	9367
Lamp1	LAMP1 (D2D11) XP rabbit MAb	CST	9091
Dynein	DYNC1H1 rabbit polyclonal antibody	Proteintech	12345-1-AP
Vimentin	Vimentin (D21H3) XP rabbit MAb	CST	5741
β -actin	β -Actin (C4) antibody	Santa Cruz Biotechnology	SC-47778
Secondary antibody	Anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat affinity-isolated antibody	Sigma	A0545
	Anti-mouse IgG (whole molecule)-peroxidase antibody produced in rabbit IgG fraction of antiserum, buffered aqueous solution	Sigma	A9044
	CoraLite488-conjugated goat anti-mouse IgG(H+L)	Proteintech	SA00013-1
	Alexa Fluor 488 AffiniPure goat anti-rabbit IgG (H+L)	Fcmacs	FMS-Rbaf48801
	Alexa Fluor 647 AffiniPure goat anti-rabbit/mouse IgG (H+L)	Fcmacs	FMS-Rbaf64701/FMS-
	CoraLite594-conjugated goat anti-rabbit/mouse IgG(H+L)	Proteintech	SA00013-4, SA00013-3

^aMAb, monoclonal antibody.

that CSFV moved rapidly along microtubules toward the interior region of cells, which is consistent with previous studies.

The present study provides for the first time the essential roles of microfilaments and microtubules in CSFV entry and trafficking in porcine epithelial (PK-15) cells. Multiple factors may regulate different steps of viral internalization. Our findings suggest the involvement of the cytoskeleton in the internalization of cargo and the initial formation of endosomes during clathrin-mediated endocytosis. The molecular mechanisms elucidated in this study broaden our understanding of the pathways required for CSFV entry and facilitate new strategies for combating CSF.

MATERIALS AND METHODS

Virus and cells. The virulent CSFV Shimen strain (GenBank accession number [AF092448](#)) was obtained from the National Institute of Veterinary Drug Control (Beijing, China). Porcine kidney (PK-15) cells and porcine alveolar macrophages (3D4/21) were maintained in Dulbecco's modified essential medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen), 0.2% NaHCO₃, 100 μ g/ml streptomycin, and 100 IU/ml penicillin (Gibco, Invitrogen) at 37°C with 5% CO₂.

Antibodies and inhibitors. Mouse monoclonal anti-E2 of CSFV antibody (WH303) was a gift from Qin Wang (National Institute of Veterinary Drug Control). All of the other antibodies and the inhibitors used for this study were obtained from commercial vendors, and details are given in Tables 1 and 2.

Cell viability assay. PK-15 cells were seeded in a 96-well plate (10⁴ cells/well) and treated with different concentrations of drugs for 24 h. The cytotoxic effect of the drugs on PK-15 cells was evaluated using a Cell Counting Kit-8 (catalog no. abs50003; Absin Bioscience, Inc.) The reagent's fluorescence was measured with a fluorescence microplate reader after 4 h of incubation in 37°C. No cytotoxicity was observed in cells treated with the indicated concentrations of the drug duplexes.

Cell infection and drug treatments. To test the effects of these drugs on CSFV infection, PK-15 cells were seeded in 24-well plates. After reaching 90% confluence, the cells were treated with the indicated drug concentrations for 1 h at 37°C. For binding and entry experiments, cells pretreated with different drugs were inoculated at an MOI of 10 in the presence of the drug at 4°C for 1 h and then shifted to 37°C.

TABLE 2 Inhibitors and drugs used in this study

Inhibitor	Specificity	Supplier
Cytochalasin D	Actin-depolymerizing agent	Cayman Chemical
Latrunculin A	Sequesters actin monomers	Cayman Chemical
Jasplakinolide	Stabilizes actin polymers	ChemCruz
Rhosin	Specific RhoA subfamily Rho GTPases inhibitor	Calbiochem
NSC23766	Inhibitor of Rac1 activation	TargetMol
ML141	Inhibitor of Cdc42 GTPase	TargetMol
Y-27632	ATP-competitive inhibitor of ROCK-I and ROCK-II	MCE
IPA-3	Prevents PAK-1 activation (irreversible)	MCE
U0126	MEK1/2 inhibitor	MCE
OSU-03012	PDK-1 inhibitor, PI3K/AKT pathway inhibitor	MCE
LY294002	Reversible PI3K inhibitor	Cayman Chemical
AG-1478	EGFR-specific inhibitor	MCE
BMS-5	LIMK inhibitor	MCE
Nocodazole	Microtubule inhibitor	Cayman Chemical
Ciliobrevin D	Specific inhibitor of AAA+ ATPase motor cytoplasmic dynein	MCE

C At 0 (binding) or 1 (entry) hpi, the cells were lysed by three freeze-thaw cycles, total RNA was extracted using TRIzol reagent, and viral RNA was measured using RT-qPCR as described previously. Data are presented as $2^{-\Delta\Delta C_T}$ values (where C_T is the threshold cycle) from quadruplicate samples. For the replication experiments, cells pretreated with the indicated drugs were infected at an MOI of 1 in the presence of the drug at 4°C for 1 h. Cells were washed with phosphate-buffered saline (PBS) and incubated in maintenance medium without the drug for 24 h at 37°C. Cells were lysed, the total RNA was extracted, and the viral RNA was measured as described above.

siRNA transfection. For RNA knockdown, PK-15 cells were grown to 50% confluence on coverslip dishes and transfected with 100 nM siRNA using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The siRNA duplexes used in the study are as follows: PI3K (catalog no. sc-62802; Santa Cruz Biotechnology), Dynein (catalog no. sc-43738; Santa Cruz Biotechnology), and the negative-control siRNA (catalog no. sc-37007; Santa Cruz Biotechnology). Other siRNA duplexes targeting RhoA, Rac1, Cdc42, EGFR, and vimentin were designed and synthesized by Shanghai GenePharma Biotechnology Co., Ltd. The sequences of these siRNA duplexes used in this study were as shown in Table 3. At 48 h post-transfection, cells were infected with CSFV; at 1 hpi or 24 hpi, virus entry or replication was measured by RT-qPCR or Western blotting.

RT-qPCR. Total RNA was extracted from the infected PK-15 cells using TRIzol reagent (Invitrogen, USA), and viral RNA was measured using RT-qPCR as described previously (71). Data are presented as $2^{-\Delta\Delta C_T}$ (where C_T is the threshold cycle) from quadruplicate samples.

Confocal microscopy. PK-15 cells grown on dishes were infected with CSFV (MOI = 10) at 4°C for 1 h and rinsed, then shifted to 37°C for the indicated time points. After incubation, the monolayers were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100. Then cells were stained with the respective specific antibodies. After washed three times with PBS, cells were stained with corresponding secondary antibodies. Then cells were stained with fluorescein isothiocyanate (FITC)-phalloidin probe dye, Tubulin Tracker red, or 4',6-diamidino-2-phenylindole (DAPI). Finally, the colocalization coefficients were calculated using the professional quantitative colocalization analysis software included with a Nikon A1 confocal microscope, and expressed as Pearson's correlation coefficients.

Western blotting. PK-15 cells grown on dishes were infected with CSFV (MOI = 10) at 4°C for 1 h and rinsed, and shifted to 37°C for the indicated time points. After incubation, cells were washed three times with ice-cold PBS and then lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (R0020; Solarbio) for 30 min at 4°C. Lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4°C. A 120-μl aliquot of the supernatant was removed from all samples for later use, then resuspended in a 5× SDS loading buffer. Proteins in the lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated antibodies. β-Actin was used as a loading control. To determine levels of indicated proteins, the corresponding protein/β-actin quantity was used to calculate the

TABLE 3 siRNA duplexes used in this study

Primer	Sequence (5'–3')	Use
siRhoA	GGCAAGACUUGUUUGCUCATT	RhoA interference RNA
siRac1	GGAGAUCGGUGCUGUGAAATT	Rac1 interference RNA
siCdc42	AGAGGAUUAUGACAGAUUATT	Cdc42 interference RNA
siEGFR	CGCUGGAGGAGAAGAAAGUTT	EGFR interference RNA
siVim	UAGUGUCUUGGUAGUUAGCTT	Vimentin interference RNA

grayscale using ImageJ 7.0 software.

Statistical analysis. All data were presented as means $\pm$ standard deviations (SD), as indicated. Student's *t* test was used to compare the data from pairs of treated and untreated groups. Asterisks in the figures indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). All statistical analyses and calculations were performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA).

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REFERENCES

- Moenning V. 1990. Pestiviruses: a review. *Vet Microbiol* 23:35–54. [https://doi.org/10.1016/0378-1135\(90\)90135-i](https://doi.org/10.1016/0378-1135(90)90135-i).
- Schweizer M, Peterhans E. 2014. Pestiviruses. *Annu Rev Anim Biosci* 2:141–163. <https://doi.org/10.1146/annurev-animal-022513-114209>.
- Meyers G, Thiel HJ, Rumenapf T. 1996. Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. *J Virol* 70:1588–1595. <https://doi.org/10.1128/JVI.70.3.1588-1595.1996>.
- Kuta A, Woźniakowski G, Polak MP. 2015. Cross-priming amplification for detection of bovine viral diarrhoea virus species 1 and 2. *J Appl Microbiol* 119:632–639. <https://doi.org/10.1111/jam.12859>.
- Passler T, Riddell KP, Edmondson MA, Chamorro MF, Neill JD, Brodersen BW, Walz HL, Galik PK, Zhang Y, Walz PH. 2014. Experimental infection of pregnant goats with bovine viral diarrhoea virus (BVDV) 1 or 2. *Vet Res* 45:38. <https://doi.org/10.1186/1297-9716-45-38>.
- Mao L, Liu X, Li W, Yang L, Zhang W, Jiang J. 2015. Characterization of one sheep border disease virus in China. *Virol J* 12:15. <https://doi.org/10.1186/s12985-014-0217-9>.
- Tautz N, Tews BA, Meyers G. 2015. The molecular biology of pestiviruses. *Adv Virus Res* 93:47–160. <https://doi.org/10.1016/bs.aivir.2015.03.002>.
- Beer M, Goller KV, Staubach C, Blome S. 2015. Genetic variability and distribution of classical swine fever virus. *Anim Health Res Rev* 16:33–39. <https://doi.org/10.1017/S1466252315000109>.
- Sun SQ, Yin SH, Guo HC, Jin Y, Shang YJ, Liu XT. 2013. Genetic typing of classical swine fever virus isolates from China. *Transbound Emerg Dis* 60:370–375. <https://doi.org/10.1111/j.1865-1682.2012.01346.x>.
- An DJ, Lim SI, Choe S, Kim KS, Cha RM, Cho IS, Song JY, Hyun BH, Park BK. 2018. Evolutionary dynamics of classical swine fever virus in South Korea: 1987–2017. *Vet Microbiol* 225:79–88. <https://doi.org/10.1016/j.vetmic.2018.09.020>.
- Chen N, Huang Y, Ye M, Li S, Xiao Y, Cui B, Zhu J. 2019. Co-infection status of classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circoviruses (PCV2 and PCV3) in eight regions of China from 2016 to 2018. *Infect Genet Evol* 68:127–135. <https://doi.org/10.1016/j.meegid.2018.12.011>.
- Xing C, Lu Z, Jiang J, Huang L, Xu J, He D, Wei Z, Huang H, Zhang H, Murong C, Tu C, Gong W. 2019. Sub-subgenotype 2.1c isolates of classical swine fever virus are dominant in Guangdong province of China, 2018. *Infect Genet Evol* 68:212–217. <https://doi.org/10.1016/j.meegid.2018.12.029>.
- Isoda N, Baba K, Ito S, Ito M, Sakoda Y, Makita K. 2020. Dynamics of classical swine fever spread in wild boar in 2018–2019, Japan. *Pathogens* 9:119. <https://doi.org/10.3390/pathogens9020119>.
- Schliwa M, van Blerkom J. 1981. Structural interaction of cytoskeletal components. *J Cell Biol* 90:222–235. <https://doi.org/10.1083/jcb.90.1.222>.
- Fletcher DA, Mullins RD. 2010. Cell mechanics and the cytoskeleton. *Nature* 463:485–492. <https://doi.org/10.1038/nature08908>.
- Pollard TD, Cooper JA. 2009. Actin, a central player in cell shape and movement. *Science* 326:1208–1212. <https://doi.org/10.1126/science.1175862>.
- Etienne-Manneville S. 2013. Microtubules in cell migration. *Annu Rev Cell Dev Biol* 29:471–499. <https://doi.org/10.1146/annurev-cellbio-101011-155711>.
- Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J. 1997. ER-to-Golgi transport visualized in living cells. *Nature* 389:81–85. <https://doi.org/10.1038/38001>.
- Reck-Peterson SL, Redwine WB, Vale RD, Carter AP. 2018. The cytoplasmic dynein transport machinery and its many cargoes. *Nat Rev Mol Cell Biol* 19:382–398. <https://doi.org/10.1038/s41580-018-0004-3>.
- Hill TL, Kirschner MW. 1982. Subunit treadmill of microtubules or actin in the presence of cellular barriers: possible conversion of chemical free energy into mechanical work. *Proc Natl Acad Sci U S A* 79:490–494. <https://doi.org/10.1073/pnas.79.2.490>.
- Tabony J, Job D. 1990. Spatial structures in microtubular solutions requiring a sustained energy source. *Nature* 346:448–451. <https://doi.org/10.1038/346448a0>.
- Matis M, Russler-Germain DA, Hu Q, Tomlin CJ, Axelrod JD. 2014. Microtubules provide directional information for core PCP function. *Elife* 3:e02893. <https://doi.org/10.7554/eLife.02893>.
- Frieden BR, Gatenby RA. 2019. Signal transmission through elements of the cytoskeleton form an optimized information network in eukaryotic cells. *Sci Rep* 9:6110. <https://doi.org/10.1038/s41598-019-42343-2>.
- Povea-Cabello S, Oropesa-Avila M, de la Cruz-Ojeda P, Villanueva-Paz M, de la Mata M, Suarez-Rivero JM, Alvarez-Cordoba M, Villalon-Garcia I, Cotan D, Ybot-Gonzalez P, Sanchez-Alcazar JA. 2017. Dynamic reorganization of the cytoskeleton during apoptosis: the two coffins hypothesis. *Int J Mol Sci* 18:2393. <https://doi.org/10.3390/ijms18112393>.
- Cortese M, Goellner S, Acosta EG, Neufeldt CJ, Oleksiuk O, Lampe M, Haselmann U, Funaya C, Schieber N, Ronchi P, Schorb M, Pruunsild P, Schwab Y, Chatel-Chaix L, Ruggieri A, Bartenschlager R. 2017. Ultrastructural characterization of Zika virus replication factories. *Cell Rep* 18:2113–2123. <https://doi.org/10.1016/j.celrep.2017.02.014>.
- Zhang LJ, Xia L, Liu SL, Sun EZ, Wu QM, Wen L, Zhang ZL, Pang DW. 2018. A “driver switchover” mechanism of influenza virus transport from microfilaments to microtubules. *ACS Nano* 12:474–484. <https://doi.org/10.1021/acs.nano.7b06926>.
- Li Q, Li W, Yin W, Guo J, Zhang ZP, Zeng D, Zhang X, Wu Y, Zhang XE, Cui Z. 2017. Single-particle tracking of human immunodeficiency virus type 1 productive entry into human primary macrophages. *ACS Nano* 11:3890–3903. <https://doi.org/10.1021/acs.nano.7b00275>.
- Hu W, Zhu L, Yang X, Lin J, Yang Q. 2016. The epidermal growth factor receptor regulates cofilin activity and promotes transmissible gastroenteritis virus entry into intestinal epithelial cells. *Oncotarget* 7:12206–12221. <https://doi.org/10.18632/oncotarget.7723>.
- Zheng K, Xiang Y, Wang X, Wang Q, Zhong M, Wang S, Wang X, Fan J, Kitazato K, Wang Y. 2014. Epidermal growth factor receptor-PI3K signaling controls cofilin activity to facilitate herpes simplex virus 1 entry into neuronal cells. *mBio* 5:e00958-13–e00913. <https://doi.org/10.1128/mBio.00958-13>.
- Lv X, Li Z, Guan J, Hu S, Zhang J, Lan Y, Zhao K, Lu H, Song D, He H, Gao F, He W. 2018. Porcine hemagglutinating encephalomyelitis virus activation of the integrin $\alpha 5 \beta 1$ -FAK-cofilin pathway causes cytoskeletal rearrangement to promote its invasion of N2a cells. *J Virol* 93:e01736-18. <https://doi.org/10.1128/JVI.01736-18>.
- Xu Q, Cao M, Song H, Chen S, Qian X, Zhao P, Ren H, Tang H, Wang Y, Wei Y, Zhu Y, Qi Z. 2016. Caveolin-1-mediated Japanese encephalitis virus entry requires a two-step regulation of actin reorganization. *Future Microbiol* 11:1227–1248. <https://doi.org/10.2217/fmb-2016-0002>.
- Baktash Y, Madhav A, Collier KE, Randall G. 2018. Single particle imaging of polarized hepatoma organoids upon hepatitis C virus infection reveals an ordered and sequential entry process. *Cell Host Microbe* 23:382–394. <https://doi.org/10.1016/j.chom.2018.02.005>.
- Martin-Acebes MA, Blazquez AB, Saiz JC. 2015. Reconciling West Nile virus with the autophagic pathway. *Autophagy* 11:861–864. <https://doi.org/10.1080/15548627.2015.1037062>.
- Chu JJ, Ng ML. 2004. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J Virol* 78:10543–10555. <https://doi.org/10.1128/JVI.78.19.10543-10555.2004>.
- Chang L, Goldman RD. 2004. Intermediate filaments mediate cytoskeletal crosstalk. *Nat Rev Mol Cell Biol* 5:601–613. <https://doi.org/10.1038/nrm1438>.
- Shi BJ, Liu CC, Zhou J, Wang SQ, Gao ZC, Zhang XM, Zhou B, Chen PY. 2016. Entry of classical swine fever virus into PK-15 cells via a pH-, dynamin-, and cholesterol-dependent, clathrin-mediated endocytic pathway

- that requires Rab5 and Rab7. *J Virol* 90:9194–9208. <https://doi.org/10.1128/JVI.00688-16>.
37. Zhang YN, Liu YY, Xiao FC, Liu CC, Liang XD, Chen J, Zhou J, Baloch AS, Kan L, Zhou B, Qiu HJ. 2018. Rab5, Rab7, and Rab11 are required for caveola-dependent endocytosis of classical swine fever virus in porcine alveolar macrophages. *J Virol* 92:e00797-18. <https://doi.org/10.1128/JVI.00797-18>.
 38. Wioland H, Guichard B, Senju Y, Myram S, Lappalainen P, Jegou A, Romet-Lemonne G. 2017. ADF/cofilin accelerates actin dynamics by severing filaments and promoting their depolymerization at both ends. *Curr Biol* 27:1956–1967.e7. <https://doi.org/10.1016/j.cub.2017.05.048>.
 39. Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z. 2002. Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 35:121–133. [https://doi.org/10.1016/s0896-6273\(02\)00758-4](https://doi.org/10.1016/s0896-6273(02)00758-4).
 40. Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K. 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393:809–812. <https://doi.org/10.1038/317135>.
 41. Narumiya S, Thumkeo D. 2018. Rho signaling research: history, current status and future directions. *FEBS Lett* 592:1763–1776. <https://doi.org/10.1002/1873-3468.13087>.
 42. Hall A. 2005. Rho GTPases and the control of cell behaviour. *Biochem Soc Trans* 33:891–895. <https://doi.org/10.1042/BST20050891>.
 43. Etienne-Manneville S, Hall A. 2002. Rho GTPases in cell biology. *Nature* 420:629–635. <https://doi.org/10.1038/nature01148>.
 44. Ridley AJ. 2001. Rho proteins: linking signaling with membrane trafficking. *Traffic* 2:303–310. <https://doi.org/10.1034/j.1600-0854.2001.002005303.x>.
 45. Julian L, Olson MF. 2014. Rho-associated coiled-coil containing kinases (ROCK): structure, regulation, and functions. *Small GTPases* 5:e29846. <https://doi.org/10.4161/sgtp.29846>.
 46. Eby JJ, Holly SP, van Drogen F, Grishin AV, Peter M, Drubin DG, Blumer KJ. 1998. Actin cytoskeleton organization regulated by the PAK family of protein kinases. *Curr Biol* 8:967–970. [https://doi.org/10.1016/s0960-9822\(98\)00398-4](https://doi.org/10.1016/s0960-9822(98)00398-4).
 47. Wee P, Wang Z. 2017. Epidermal growth factor receptor cell proliferation signaling pathways. *Cancers (Basel)* 9:52. <https://doi.org/10.3390/cancers9050052>.
 48. Wang X, Huang DY, Huang SM, Huang ES. 2005. Integrin $\alpha v \beta 3$ is a coreceptor for human cytomegalovirus. *Nat Med* 11:515–521. <https://doi.org/10.1038/nm1236>.
 49. Weller ML, Amornphimoltham P, Schmidt M, Wilson PA, Gutkind JS, Chiorini JA. 2010. Epidermal growth factor receptor is a co-receptor for adeno-associated virus serotype 6. *Nat Med* 16:662–664. <https://doi.org/10.1038/nm.2145>.
 50. Chen SW, Himeno M, Kou Y, Sugiyama M, Nishitsuji H, Mizokami M, Shimotohno K, Miyajima A, Kido T. 2020. Modulation of hepatitis B virus infection by epidermal growth factor secreted from liver sinusoidal endothelial cells. *Sci Rep* 10:14349. <https://doi.org/10.1038/s41598-020-71453-5>.
 51. Hancock WO. 2014. Bidirectional cargo transport: moving beyond tug of war. *Nat Rev Mol Cell Biol* 15:615–628. <https://doi.org/10.1038/nrm3853>.
 52. Xiang X, Qiu R, Yao X, Arst HN, Jr, Penalva MA, Zhang J. 2015. Cytoplasmic dynein and early endosome transport. *Cell Mol Life Sci* 72:3267–3280. <https://doi.org/10.1007/s00018-015-1926-y>.
 53. Strelkov SV, Herrmann H, Aebi U. 2003. Molecular architecture of intermediate filaments. *Bioessays* 25:243–251. <https://doi.org/10.1002/bies.10246>.
 54. Foo KY, Chee HY. 2015. Interaction between flavivirus and cytoskeleton during virus replication. *Biomed Res Int* 2015:427814. <https://doi.org/10.1155/2015/427814>.
 55. Enjuanes L, Almazan F, Sola I, Zuniga S. 2006. Biochemical aspects of coronavirus replication and virus-host interaction. *Annu Rev Microbiol* 60:211–230. <https://doi.org/10.1146/annurev.micro.60.080805.142157>.
 56. Miranda-Saksena M, Denes CE, Diefenbach RJ, Cunningham AL. 2018. Infection and transport of herpes simplex virus type 1 in neurons: role of the cytoskeleton. *Viruses* 10:92. <https://doi.org/10.3390/v10020092>.
 57. Bravo-Cordero JJ, Magalhaes MA, Eddy RJ, Hodgson L, Condeelis J. 2013. Functions of cofilin in cell locomotion and invasion. *Nat Rev Mol Cell Biol* 14:405–415. <https://doi.org/10.1038/nrm3609>.
 58. Pope BJ, Zierler-Gould KM, Kuhne R, Weeds AG, Ball LJ. 2004. Solution structure of human cofilin: actin binding, pH sensitivity, and relationship to actin-depolymerizing factor. *J Biol Chem* 279:4840–4848. <https://doi.org/10.1074/jbc.M310148200>.
 59. Chaikereeratsak V, Khanna K, Nguyen KT, Sugie J, Egan ME, Erb ML, Vavilina A, Nonejuie P, Nieweglowska E, Pogliano K, Agard DA, Villa E, Pogliano J. 2019. Viral capsid trafficking along treadmill tubulin filaments in bacteria. *Cell* 177:1771–1780.e12. <https://doi.org/10.1016/j.cell.2019.05.032>.
 60. Greene W, Gao SJ. 2009. Actin dynamics regulate multiple endosomal steps during Kaposi's sarcoma-associated herpesvirus entry and trafficking in endothelial cells. *PLoS Pathog* 5:e1000512. <https://doi.org/10.1371/journal.ppat.1000512>.
 61. Walsh D, Naghavi MH. 2019. Exploitation of cytoskeletal networks during early viral infection. *Trends Microbiol* 27:39–50. <https://doi.org/10.1016/j.tim.2018.06.008>.
 62. Zhang Y, Gao W, Li J, Wu W, Jiu Y. 2019. The role of host cytoskeleton in flavivirus infection. *Virol Sin* 34:30–41. <https://doi.org/10.1007/s12250-019-00086-4>.
 63. Kast DJ, Dominguez R. 2017. The cytoskeleton-autophagy connection. *Curr Biol* 27:R318–R326. <https://doi.org/10.1016/j.cub.2017.02.061>.
 64. Naghavi MH, Walsh D. 2017. Microtubule regulation and function during virus infection. *J Virol* 91:e00538-17. <https://doi.org/10.1128/JVI.00538-17>.
 65. Hoornweg TE, Bouma EM, van de Pol DPI, Rodenhuis-Zybert IA, Smit JM. 2020. Chikungunya virus requires an intact microtubule network for efficient viral genome delivery. *PLoS Negl Trop Dis* 14:e0008469. <https://doi.org/10.1371/journal.pntd.0008469>.
 66. Liu CC, Zhang YN, Li ZY, Hou JX, Zhou J, Kan L, Zhou B, Chen PY. 2017. Rab5 and Rab11 are required for clathrin-dependent endocytosis of Japanese encephalitis virus in BHK-21 cells. *J Virol* 91:e01113-17. <https://doi.org/10.1128/JVI.01113-17>.
 67. Bhabha G, Johnson GT, Schroeder CM, Vale RD. 2016. How dynein moves along microtubules. *Trends Biochem Sci* 41:94–105. <https://doi.org/10.1016/j.tibs.2015.11.004>.
 68. Carnes SK, Zhou J, Aiken C. 2018. HIV-1 engages a dynein-dynactin-BICD2 complex for infection and transport to the nucleus. *J Virol* 92:e00358-18. <https://doi.org/10.1128/JVI.00358-18>.
 69. Osseman Q, Gallucci L, Au S, Cazenave C, Berdancé E, Blondot ML, Cassany A, Begu D, Ragues J, Aknin C, Sominskaya I, Dishlers A, Rabe B, Anderson F, Pante N, Kann M. 2018. The chaperone dynein LL1 mediates cytoplasmic transport of empty and mature hepatitis B virus capsids. *J Hepatol* 68:441–448. <https://doi.org/10.1016/j.jhep.2017.10.032>.
 70. Dharan A, Campbell EM. 2018. Role of microtubules and microtubule-associated proteins in HIV-1 infection. *J Virol* 92:e00085-18. <https://doi.org/10.1128/JVI.00085-18>.
 71. He DN, Zhang XM, Liu K, Pang R, Zhao J, Zhou B, Chen PY. 2014. *In vitro* inhibition of the replication of classical swine fever virus by porcine Mx1 protein. *Antiviral Res* 104:128–135. <https://doi.org/10.1016/j.antiviral.2014.01.020>.