White spot syndrome virus benefits from endosomal trafficking, substantially facilitated by a valosin-containing protein, to escape autophagic elimination and propagate in crustacean *Cherax quadricarinatus*

Chuang Meng[1], Ling-Ke Liu[1], Dong-Li Li[1], Rui-Lin Gao[1], Wei-Wei Fan[1], Ke-Jian Wang[1], Han-Ching Wang[3] and Hai-Peng Liu[1, 2#]

[1] State Key Laboratory of Marine Environmental Science; State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology; College of Ocean and Earth Sciences, Xiamen University; Xiamen, 361102, Fujian, China.

[2] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology (Qingdao), China.

[3] Department of Biotechnology and Bioindustry Sciences, College of Biosciences and Biotechnology, National Cheng Kung University, Tainan, Taiwan

[#] Corresponding author

Dr. Hai-Peng Liu; Mailing address: College of Ocean and Earth Sciences, Xiamen University; Xiamen, 361102, Fujian. E-mail address: haipengliu@xmu.edu.cn.

Running Title: CqVCP facilitates WSSV trafficking for infection

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ABSTRACT

As the most severely lethal viral pathogen for crustaceans both in brackish water and freshwater, the white spot syndrome virus (WSSV) has a mechanism of infection that remains largely unknown, which profoundly limits the control of WSSV disease. By using a hematopoietic tissue (Hpt) stem cell culture from red claw crayfish *Cherax quadricarinatus* suitable for WSSV propagation *in vitro*, the intracellular trafficking of live WSSV was determined for the first time via live-cell imaging, in which the acidic pH-dependent endosomal environment was a prerequisite for WSSV fusion. When the acidic pH within the endosome was alkalized by chemicals, the intracellular WSSV virions were detained in dysfunctional endosomes, resulting in appreciable blocking of the viral infection. Furthermore, disrupted valosin-containing protein (*Cq*VCP) activity resulted in considerable aggregation of endocytic WSSV virions in the disordered endosomes, which subsequently recruited autophagosomes, likely by binding to *Cq*GABARAP via *Cq*VCP, to eliminate the aggregated virions within the dysfunctional endosomes. Importantly, both autophagic sorting and the degradation of intracellular WSSV virions were clearly enhanced in the Hpt cells with increased autophagic activity, demonstrating that autophagy played a defensive role against the WSSV infection. Intriguingly, most of the endocytic WSSV virions were directed to the endosomal delivery system facilitated by *Cq*VCP activity such that they avoided autophagy degradation and successfully delivered the viral genome into Hpt cell nucleus, which was followed by the propagation of progeny virions. These findings will benefit anti-WSSV target design against the most severe viral disease currently affecting farmed crustaceans.

45 **IMPORTANCE**

46 White spot disease is currently the most devastating viral disease in farmed
47 crustaceans, such as shrimp and crayfish, which has resulted in a severe ecological
48 problem for both brackish water and fresh water aquaculture areas worldwide. The
49 efficient antiviral control against WSSV disease is still lacking due to our limited
50 knowledge of its pathogenesis. Importantly, research on the WSSV infection
51 mechanism is also quite meaningful for the elucidation of viral pathogenesis and
52 virus-host coevolution, as WSSV is one of the largest animal viruses in terms of
53 genome size which infects only crustaceans. Here, we found that most of the
54 endocytic WSSV virions were directed to the endosomal delivery system strongly
55 facilitated by *Cq*VCP, such that they avoided autophagic degradation and successfully
56 delivered the viral genome into the Hpt cell nucleus for propagation. Our data point to
57 a virus-sorting model that might also explain the escape of other enveloped DNA
58 viruses.

59

INTRODUCTION

White spot disease caused by white spot syndrome virus (WSSV) is the most devastating viral disease in farmed crustaceans since its first outbreak in the 1990s in Southeast Asia; it infects only crustaceans, those in both brackish water and fresh water, including shrimp, crayfish, crab and more than one hundred other crustacean species (1). WSSV, originally found from marine shrimp, has spread from seawater to freshwater aquaculture areas in China and United States of America (1, 2); for example, most wild freshwater swamp crayfish *Procambarus clarkii* in China carry this virus, causing a severe ecological problem for both brackish water and freshwater aquaculture areas around the world. WSSV is the sole member of the Whispovirus genus in the *Nimaviridae* family, which is composed of envelope, nucleocapsid and double-stranded DNA. The complete genome sequence of WSSV, one of the largest among animal-infecting viruses, is more than ~300 Kb (3). Additionally, a suitable cell line for the efficient propagation of progeny virions *in vitro* is lacking, which makes the genetic manipulation and reconstruction of WSSV currently unattainable. Therefore, the infection mechanism and life cycle of WSSV remain largely unknown. The major viral proteins of the envelope, as well as those of nucleocapsid, in WSSV have been annotated (4). In particular, envelope proteins have been found to be important for WSSV infection, as these proteins are the first viral molecules to interact with host cells during the initiation stage of infection, and therefore, they play critical roles in cell targeting and triggering the cellular response. However, the details of envelope fusion (5) and subsequent nucleocapsid release are largely unknown,

82 which severely hampers disease control against WSSV, considering that the release of
83 nucleocapsids is a prerequisite for the successful delivery of viral genomic DNA into
84 the host cell nucleus, which is also a suitable target for antiviral design.

85 It is well known that enveloped DNA viruses are usually endocytosed after
86 membrane internalization activated by viral receptor binding, which forms endocytic
87 vesicles that fuse with early endosomes and then mature into late endosomes (6).
88 Acidification-driven envelope fusion with the endosome membrane is a prerequisite
89 for the successful penetration and further delivery of the viral genome into the host
90 cell nucleus. In addition, the vacuolar V-ATPase is critical for regulating the gradually
91 decreasing pH, from approximately 7.4 to 6.2, in early endosomes and to
92 approximately 5.5 in late endosomes (7). Subsequently, the nucleocapsid containing
93 the viral genome penetrates the cytosol, and the viral genome DNA enclosed by the
94 nucleocapsid is transported via intracellular trafficking machinery, such as the
95 endosomal delivery system and the cytoskeleton system, into the host cell nucleus (8),
96 where the viral genome undergoes transcription and replication. Thus, endosomal
97 acidification plays a critical role in the successful infection of most enveloped DNA
98 viruses such as baculovirus (9) and African swine fever virus (ASFV) (10). Recently,
99 multiple endocytic routes, such as those of clathrin-mediated endocytosis (CME),
100 caveolae-mediated endocytosis and micropinocytosis, were found to be activated for
101 WSSV entry into host cells (11-13). Among other researchers, we found that the CME
102 pathway played a key role in WSSV entry (12), suggesting that the subsequent
103 intracellular trafficking of WSSV via endosomes might be essential for successful

104 infection, considering that CME vesicles containing viral cargos are usually
105 subsequently fused with endosomes. However, the details of WSSV intracellular
106 trafficking after its internalization by the host cell membrane remain largely unknown,
107 particularly those of the mechanisms associated with intracellular trafficking, fusion
108 and uncoating of WSSV. Therefore, it is important to elucidate the endocytic transport
109 of CME vesicles containing WSSV virions, *i.e.*, the main pathway for WSSV entry,
110 the subsequent fusion of WSSV with endosomes (or not) and nucleocapsid uncoating
111 followed by delivery of the viral genome into the host cell nucleus, which may
112 provide novel targets against WSSV disease.

113 The valosin-containing protein (VCP/p97) is an evolutionarily conserved ATPase
114 associated with various cellular activities, such as membrane fusion (14), proteasome
115 degradation as a segregase (15), endosomal sorting (16) and autophagy (17). In
116 addition, VCP/p97 has been proposed to be involved with the acidification of acidic
117 organelles, such as endosomes and autophagosomes, as mediated by
118 V-ATPase-induced hydrolysis of ATP in vacuolar compartments, which is essential for
119 the acidification of the endosome compartment. Either overexpression of mutant VCP
120 proteins or chemical inhibition of VCP activity decreased the acidity of the
121 endolysosome in HEK293 cells, and the substrates were detained within the enlarged
122 endosome (16). Interestingly, VCP was reported to play a critical role in endocytic
123 transport as well as in the maturation of virus-loaded endosomes, including those in
124 host cells infected with enveloped viruses such as Sindbis virus (18) and infectious
125 bronchitis virus (IBV) (19), in which IBV accumulated in the immature endosome,

126 unable to transfer capsids into the cytosol, by inhibiting either VCP or V-ATPase
127 activity (19). Recently, the endocytic nucleocapsids of enveloped baculovirus
128 *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) were found to be
129 trapped within cytoplasmic vacuoles after inhibition of VCP/TER94 in Sf9 cells (20).
130 Additionally, VCP was required for replication of influenza virus (21) and human
131 cytomegalovirus (22). Thus, these findings suggest that VCP participates in vacuolar
132 trafficking or replication of some envelope viruses. To the best of our knowledge,
133 however, little is known about VCP-related viral infection in crustaceans. A VCP from
134 shrimp *Penaeus vannamei* has been shown to be involved in WSSV pathogenesis, as
135 indicated by a protein expression profiling analysis (23), but no further functional
136 details have been reported. In addition, we found a partial transcript of *VCP* (*CqVCP*)
137 expressed 12 hpi *in vitro* by searching a cDNA library of hematopoietic tissue (Hpt)
138 cell cultures from red claw crayfish *Cherax quadricarinatus* post-WSSV infection
139 (our unpublished data). These findings suggest that VCP likely plays a role during
140 WSSV infection in crustaceans. However, the mechanism by which VCP regulates
141 viral infection in crustaceans is unknown.

142 Autophagy and its machinery are related to various biological processes at both
143 the cellular and physiological levels. In particular, the highly conserved intracellular
144 autophagy-dependent degradation system plays an important role in the immune
145 defensive system of eukaryotes (24). For example, autophagy, reflecting xenophagy in
146 this study, is activated by immunological signals and directly targets invading
147 pathogens, such as viruses (25), in a series of processes that usually involves selective

148 recognition, engulfment, trafficking and subsequent elimination upon
149 autophagosome-lysosome fusion (26). We previously reported that WSSV infection
150 induced autophagy in red claw crayfish Hpt cells, in which autophagosomes
151 containing WSSV virions were clearly observed in transmission electron microscopy
152 (TEM) analysis. In particular, the enhanced autophagic activity promoted WSSV
153 entry into red claw crayfish Hpt cells, in which a γ-aminobutyric acid
154 receptor-associated protein (*Cq*GABARAP), belonging to the Atg8 subfamily,
155 facilitated the aggregation of WSSV in the cytoplasm followed by reduced viral
156 replication at an early stage of viral infection (12). Regardless of how these virions
157 were directed to the autophagy pathway, autophagy is likely involved in the
158 intracellular trafficking of WSSV, and artificially activating defensive autophagy in
159 crustaceans is critical for the elucidation of anti-WSSV responses in crustaceans. On
160 the other hand, the endosome membrane provides a protective barrier for invading
161 pathogens facing recognition by host receptors involved in selective autophagy, such
162 as p62 and NDP52 (27). Other noncanonical autophagy mechanisms, including
163 LC3-associated phagocytosis (28), LC3-associated endocytosis (29) and
164 endosome-mediated autophagy (30), may provide available strategies for eliminating
165 endosome-containing pathogens via autophagy. For instance, when
166 endosome-containing bacteria enter the cytoplasm from endosomes, the damaged
167 endomembranes recruit autophagosomes via the V-ATPase-ATG16L1 axis (31),
168 indicating that artificially activated endosome-mediated degradative autophagy may
169 be used to eliminate the pathogens detained in endosomes. Importantly, there are also

overlapping between functions in the endosome system and autophagy during endocytic trafficking and autophagosome maturation. For example, the aforementioned VCP is able to mediate the sorting and degradation of ubiquitinated cargos in endolysosomes and is selectively required for ubiquitin-dependent degradation via autophagy (32). In consideration of the role of VCP related to the viral infection described above, a functional study of VCP in crustaceans is expected to be useful for elucidating WSSV pathogenesis, and increased understanding of the tentatively coupled processes in defensive autophagy and the "hijacked" endosomal delivery system, assuming they are related, may be critical for finding novel molecular targets against WSSV infection.

In this study, the details of intracellular trafficking of WSSV, along with viral fusion, uncoating, replication and propagation regulated by *CqVCP*, were mainly determined by using a red claw crayfish Hpt cell culture that was recently shown to be suitable for WSSV propagation *in vitro* (33). The findings contribute to our understanding of WSSV pathogenesis and to promising future anti-WSSV design targets, such as *Cq*VCP.

RESULTS

Cytoplasmic trafficking and the fusion of WSSV with acidic vesicles

To examine how WSSV is intracellularly transported after entry into host cells, TEM was used to visualize the early phase of WSSV infection in Hpt cells from red claw crayfish. As shown in Fig. 1A, the WSSV virion is initially engulfed by the Hpt

192 cell membrane and endocytosed via membrane invagination (Fig. 1A-a&b), and it is
193 then transported via an endocytic vesicle (Fig. 1A-c) and delivered to the perinuclear
194 area in the Hpt cell (Fig. 1A-d). In addition, WSSV labeled with DiD fluorescence
195 dye exhibited a tendency to accumulate over time in the cytoplasm, as determined by
196 laser confocal microscopy (Fig. 1B and Supplemental Video 1A). Interestingly, the
197 intracellular WSSV virions were found to be extensively colocalized with acidic
198 vesicles, as indicated by LysoTracker dye in live cells visualized using real-time
199 imaging techniques (Fig. 1C and Supplemental Video 1B), suggesting that the
200 intracellular trafficking of WSSV virions was realized with acidic vesicles for a rather
201 longer time intervals, in which most of the endocytosed virions were shown by time
202 lapse imaging to move towards the perinuclear area of the Hpt cells (Fig. 1C, lower
203 panel).

204 When Hpt cells were simultaneously infected by both WSSV labeled with DiO in
205 the viral envelope and WSSV labeled with DiD in the viral envelope, the fluorescence
206 of DiO-WSSV and DiD-WSSV revealed a good co-localization within certain
207 vesicles (Fig. 1D), possibly acidic vesicles as shown in Fig. 1C according to real-time
208 imaging with laser confocal microscopy. In consideration to that the time series of
209 good co-localization between viral and endosomes could indicate the possible viral
210 fusion (34), this finding suggested the possible viral fusion of both DiD-WSSV and
211 DiO-WSSV envelope with the targeted vesicles, respectively, then leading to the
212 uniform distribution of the dispersed DiD and DiO fluorescent signals in the same
213 compartment in Hpt cell cytoplasm. We then speculate that the acidic environment of

the intracellular vesicle compartment might be a prerequisite for WSSV envelope fusion with the intracellular compartments, such as the acidic endosomal compartments usually necessary for enveloped virus fusion (6), needing further investigations.

To further evaluate the time course of WSSV envelope dissociation from the nucleocapsid, colocalized fluorescent spots, indicating both the viral envelope protein VP28 and nucleocapsid protein VP664, were determined by immunofluorescence assay with antibodies against VP28 and VP664, respectively. As shown in Fig. 1E, the most intense fluorescent signal for envelope protein VP28 was observed within 0.5 hpi, followed by a gradual decrease 1 hpi in the cytoplasm, and declining to a negligible level 2 hpi, likely due to protein degradation after envelope fusion with the vesicle membrane. In addition, the most intense fluorescent VP664 signal was clearly present 1 hpi, followed by a gradual decrease from 2 to 3 hpi in the cytoplasm, indicating that most of the WSSV underwent fusion within 2 hpi in the acidic vesicles in the Hpt cells. Hence, the image recordings of WSSV trafficking in live cells provided us with data, for the first time, to answer key questions with respect to WSSV infection; for example, how is the intracellular trafficking of WSSV molecularly regulated? The answer is particularly important for determining tentative targets against WSSV disease.

The acidic pH within endosomes was a prerequisite for WSSV infection

On the basis of TEM analysis, intracellular WSSV was found to be located in intracellular vesicles with multiple vesicular bodies (MVBs), the typical structure for

endosomal compartments under TEM observation (35) in all treatments (Fig. 2A-a); in particular, endocytic WSSV virions were extensively colocalized with one of the marker proteins for endosome RabGEF1 (36), which was stained by the anti-RabGEF1 antibody in the cytoplasm of the Hpt cells (Fig. 2A-b, the control cells). This result suggested that the endocytic WSSV virions were delivered into endosomal vesicles in the Hpt cells. As shown in the control cells in Fig. 2A-b, the dispersed fluorescent signals of the viral envelope protein within these vesicle organelles strongly suggested that the tentative fusion had likely occurred between the viral envelope and the membrane of the endosomal vesicles, implying the importance of endosomal vesicles for the early intracellular trafficking of WSSV.

To further determine the effect of the endosomal system, specifically, the case of acidification with maturation, on WSSV infection, the acidity of the intracellular vesicles was attenuated by pretreatment of the Hpt cells with the alkalizers chloroquine or NH_4Cl to neutralize the H^+ in the endosomes before WSSV infection. As shown in Fig. 2B-a&b, the fluorescence intensity of the acidic vesicles, including endosomes, labeled by LysoTracker for acidity and by WGA for endosomal membrane (37), was obviously decreased by the alkalization treatment, with the extent of the "pH increase" caused by NH_4Cl being greater than that caused by chloroquine. In addition, the relative size of these intracellular fluorescent vesicles was clearly increased by alkalization treatment of the Hpt cells (Fig. 2B-c). The typical ultrastructure of a MVB containing WSSV virions, was found for both alkalizer-treated cells and control cells, but enlarged vesicles, particularly those with

increased vacuolation and accumulated WSSV virions, were found only in the alkalizer-treated Hpt cells, as shown by the TEM results (Fig. 2A-a). These findings suggested that the intracellular trafficking of WSSV was likely to be disrupted by the alkalization of the Hpt cells. To further address this speculation, the colocalization of endosomes and endocytic WSSV virions was examined after alkalization of the Hpt cells. As speculated, the WSSV virions were found to be considerably accumulated and colocalized within the endosomes, as indicated by anti-RabGEF1 antibody immunostaining in the Hpt cells after alkalization but not in the control cells (Fig. 2A-b), exhibiting the significant colocalization of VP28 and RabGEF1 in the alkalized cells treated by chloroquine or NH_4Cl (Fig. 2A-c, $p<0.01$). Meanwhile, the accumulation of WSSV-containing acidic vesicles were clearly observed in the control cells, in which the WSSV virions were then delivered into endosomes for subsequent intracellular trafficking (Fig. 2C-a and Supplemental Video 2A). In contrast to the colocalization and accumulation of the WSSV virions within the endosomal vesicles, as described above (Fig. 2C-a), the internalized DiD-WSSV virions were found to be mainly isolated in the endosomal compartments, and their trafficking was delayed after alkalization of the acidic pH in the Hpt cells, with significantly vacuolated endosomal vesicles upon chloroquine treatment, as determined by using live-cell imaging (Fig. 2C-b and Supplemental Video 2B). In addition, when Hpt cells alkalized by chloroquine were infected with both DiD-WSSV and DiO-WSSV at the same time, the endocytosed DiD-WSSV continuously accumulated into enlarged intracellular vacuoles (Fig. 2C-c and

Supplemental Video 2C) but without the speculated obvious uniform dispersion of viral fluorescence within acidic vesicles, as shown in Fig. 1D. This phenomenon was also confirmed by the pretreatment of Hpt cells with another alkalizer, NH_4Cl (data not shown), followed by WSSV infection, which showed that the intracellular movement of the WSSV virions was mainly restricted to the alkalized endosomes. These data together clearly indicated that the acidic pH within endosomal compartments was critical for the proper intracellular trafficking of WSSV. Importantly, the speculated fusion of the WSSV envelope with the endosomal membrane, as indirectly indicated by the significant colocalization of the viral envelope protein VP28 and the nucleocapsid protein VP664 (4), was profoundly blocked by alkalization of the endosomal vesicles with the alkalizer chloroquine or NH_4Cl in the Hpt cells (Fig. 2D, $p<0.01$). Furthermore, the degradation of WSSV at the early infection stage, 4 hpi, as indicated by the presence of envelope protein VP28, was strongly inhibited by the alkalization of the endosomal vesicles in the Hpt cells, as an acidic environment within endosomes was a prerequisite for viral envelope fusion followed by the efficient protein degradation, which was also associated with lower intracellular pH (Fig. 2E-a). Accordingly, both the WSSV replication (Fig. 2E-b) and viral copy numbers (Fig. 2E-c) were significantly reduced in Hpt cells alkalized by chloroquine or NH_4Cl at the very late infection stage 24 hpi. These findings together indicated that the endocytosed WSSV virions were delivered into the endosomal vesicles, where the virions were efficiently “trapped” when the endosomal acidity was attenuated by alkalization in Hpt cells.

To reveal whether endosomal maturation affects WSSV fusion, in Hpt cells, the maturation of early endosomes into late endosome was blocked with the chemical inhibitor YM201636, suppressing the synthesis of PtdIns (3, 5) P2 by blocking PIKfyve enzymatic activity during late endosomal maturation (38), followed by WSSV infection. In the negative control cells, disruption of the conversion of early endosomes into late endosomes by YM-201636 treatment did not clearly inhibit WSSV fusion compared to that of the positive control cells, upon inhibition of V-ATPase activity with bafilomycin-A1 treatment (39), which profoundly blocked the acidic pH-dependent fusion of the viral envelope with the endosomal membrane in the Hpt cells. These findings were indirectly indicated by the clear colocalization of VP28 and VP664 due to the unsuccessful fusion of the envelope with the endosome membrane caused by the decreased acidification within these endosomes upon bafilomycin-A1 treatment (Fig. 2F-a). Consequently, no obvious effect on WSSV replication was observed by inhibiting the conversion of early endosomes to late endosomes (Fig. 2F-b, upper). In contrast, WSSV replication was significantly inhibited by the decreased acidity of the intracellular endosomes caused by bafilomycin-A1 against V-ATPase activity (Fig. 2F-b, lower), suggesting that the acidity of the early endosome was sufficient for WSSV fusion with the endosomal membrane that led to successful infection.

Blocking *Cq*VCP ATPase activity abolished viral fusion and the endosomal trafficking of WSSV during infection

As shown above, deficiency of the endosomal delivery system by alkalization

profoundly impaired WSSV fusion, leading to significant inhibition of WSSV infection. However, the key gene(s) involved in the regulation of the endosomal trafficking of WSSV was not clear. By searching a cDNA library of red claw crayfish Hpt cell culture post-WSSV infection, a partial transcript of *CqVCP* was found to be induced 12 hpi *in vitro* (our unpublished data), which was confirmed to be significantly induced in both mRNA transcript (Fig. 3A-a) and protein expression (Fig. 3A-b) levels by WSSV infection 1, 6 and 12 hpi *in vivo* in red claw crayfish hematopoietic tissue. However, whether the endosomal trafficking of WSSV was regulated by VCP in crustaceans remained unclear. To address this question, the complete cDNA sequence of the red claw crayfish *VCP* gene (*CqVCP*, Genbank No: 2091681) was cloned, and it contained a highly conserved motif with the typical AAA-ATPase domain of the CDC48 subfamily (40). To further determine whether WSSV infection could be affected by *Cq*VCP activity, *CqVCP* gene expression was silenced by RNA interference in the Hpt cells, in which both the transcript level ($p<0.01$, left) and the protein level of *Cq*VCP ($p<0.05$, right) were significantly decreased (Fig. 3B-a). Interestingly, both the transcription of the WSSV gene, as indicated by gene transcript of *VP28* (an approximate 80% decrease, $p<0.01$, Fig. 3B-a, left), and the degradation of the detached envelope, as indicated by the presence of the viral envelope protein VP28 (Fig. 3B-a, right, $p<0.05$), were also significantly decreased after *CqVCP* gene silencing in the Hpt cells, compared with these levels in the control cells. We speculated that these phenomena were likely caused by a blockade of WSSV fusion, *i.e.*, eliminating the prerequisite for the efficient

degradation of the envelope proteins detached from the nucleocapsid at an early infection stage, 6 hpi, in the Hpt cells. To prove the effect on WSSV infection by the disruption of *Cq*VCP activity, the ATPase activity of *Cq*VCP was blocked by the pharmacological inhibitor DBeQ, which competitively binds to the ATP-binding locus in the VCP AAA domain and then blocks the subsequent binding of ATP to VCP (41). As expected, DBeQ-bound His-*Cq*VCP showed a higher peak value than did that of the control as determined by a biolayer interferometry assay, suggesting that the DBeQ inhibitor efficiently binds to the recombinant *Cq*VCP protein (data not shown). In addition, the acidic pH within the endosomes was significantly decreased by blocking *Cq*VCP ATPase activity with DBeQ (data not shown), indicating that the acidity within endosomes was successfully disrupted in the Hpt cells by pharmacological blocking the *Cq*VCP ATPase activity. Importantly, both the gene replication (an ~90% decrease, $p<0.01$, left) and protein degradation (an ~30% decrease, $p<0.05$, right) of WSSV were also clearly inhibited upon the blocking of *Cq*VCP ATPase activity with DBeQ at an early infection stage, 6 hpi, in the Hpt cells (Fig. 3B-b), in which the inhibition of WSSV gene transcription with DBeQ was dose-dependent (data not shown). These results strongly suggested that *Cq*VCP ATPase activity was critical for the successful infection of WSSV, considering that VCP was involved in the regulation of endosomal acidification (16). As DBeQ treatment resulted in a relatively more efficient inhibition of WSSV replication (Fig. 3B-b) than was gene silencing of *CqVCP* (Fig. 3B-a), we then used DBeQ to block *Cq*VCP ATPase activity in subsequent experiments unless otherwise stated. It remains

368 unclear whether the viral fusion and intracellular trafficking of WSSV is indeed
369 affected by blocking *Cq*VCP activity. To determine WSSV trafficking in more detail,
370 particularly in relation to *Cq*VCP activity, the endosomal trafficking of WSSV was
371 dynamically examined in a time series study of live Hpt cells, in which the *Cq*VCP
372 ATPase activity was blocked with DBeQ. As shown in Fig. 3C and Supplemental
373 Video 3, the intracellular DiD-WSSV virions were found to have clearly accumulated
374 in the endosomes after *Cq*VCP ATPase activity was blocked, and more endosomal
375 vesicles containing WSSV virions were shown to be dynamically fused with each
376 other to form enlarged vesicles in the Hpt cells 3 hpi. In contrast, despite continuous
377 fusion, there was no clear accumulation in the control cells, implying that the WSSV
378 nucleocapsid enclosing the viral genome properly escaped from the endosome with
379 fully activated *Cq*VCP and was delivered after viral envelope fusion with the
380 endosomal membrane. Furthermore, the *Cq*VCP protein was shown to accumulate
381 and colocalize with RabGEF1 in the endosomes of the Hpt cells in which *Cq*VCP
382 ATPase activity had been blocked with DBeQ (Fig. 3D-a, upper). In addition, the
383 *Cq*VCP protein also clearly accumulated and colocalized with the detained WSSV
384 aggregates, as indicated by the immunostaining of VP28 in the viral envelope, which
385 colocalized within the dysfunctional endosomes (Fig. 3D-a, lower), in regarding to
386 the significant colocalization between RabGEF1 and VP28 (Fig. 3D-b, upper,
387 $p<0.001$) after *Cq*VCP ATPase activity was blocked in the Hpt cells. Most
388 importantly, the viral envelope protein VP28 and the nucleocapsid protein VP664
389 were also shown to be extensively colocalized in dysfunctional endosomes lacking

390 *Cq*VCP activity in the Hpt cells 4 hpi (Fig. 3D-b, lower, $p<0.001$), demonstrating that
391 the fusion of the viral envelope with the endosomal membrane, as well as the release
392 of the nucleocapsid, was obviously inhibited in these dysfunctional endosomes
393 because of the blocked *Cq*VCP ATPase activity. However, these phenomena were not
394 found in the control Hpt cells (Fig. 3D), in which viral envelope fusion had been
395 proposed to occur within 2 hpi, followed by further intracellular degradation of
396 detached envelope proteins, such as VP28, in agreement with the findings shown in
397 Fig. 1E. In addition, a higher number of intact WSSV virions, as indicated by ≥3
398 WSSV virions per endosomal vesicle, were confirmed to be aggregated and detained
399 in the endosomes after *Cq*VCP ATPase activity was blocked with DBeQ, compared to
400 that of control cells, as determined by TEM analysis (Fig. 3D-c), indicating that the
401 fusion between the WSSV envelope and endosomal membrane was strongly inhibited
402 upon blocking of the *Cq*VCP ATPase activity.

403 Furthermore, the efficiency of WSSV infection was also examined after
404 pharmacological blocking of *Cq*VCP ATPase activity with DBeQ in Hpt cells, in
405 which the translation of both Hpt cell protein and WSSV was blocked by pretreating
406 the Hpt cells with the protein translation inhibitor cycloheximide. As shown in Fig.
407 3E-a, the degradation of WSSV, indicated by the presence of the remaining VP28
408 envelope protein, which was considered to be mostly degraded within 2 hpi after viral
409 fusion (Fig. 1E), was found to be clearly reduced 12 hpi after the *Cq*VCP ATPase
410 activity was blocked in the Hpt cells pretreated with cycloheximide ($p<0.05$),
411 suggesting that the fusion of the WSSV envelope with the endosomal membrane was

likely inhibited by blocking *Cq*VCP ATPase activity. In the control group without cycloheximide treatment, significantly less VP28 protein was present in the DBeQ-treated cells, because the viral envelope fusion with the endosome membrane was blocked, leading to the subsequent inhibition of viral infection, as proposed above. Consistently, WSSV replication was also significantly reduced in the Hpt cells lacking *Cq*VCP activity because of DBeQ exposure in the group not treated with cycloheximide (Fig. 3E-b, $p<0.001$), which was likely caused by the unsuccessful delivery of the viral genome into the Hpt cell nucleus due to endosome dysfunction caused by inhibition of the acidification as mediated by the lack of *Cq*VCP ATPase activity. This result demonstrated that blocking *Cq*VCP ATPase activity led to WSSV aggregation within the dysfunctional endosomal vesicles in the cytoplasm and then to the inhibition of the subsequent trafficking of the nucleocapsid surrounding the viral genome to the perinuclear area for the subsequent release of the viral genome into the Hpt cell nucleus. Additionally, WSSV replication was completely blocked by cycloheximide pretreatment, abrogating viral gene transcription, such as that of VP28, for which protein translation of both host cell and viral immediate-early genes, could be deleted at 12 hpi, as tested (Fig. 3E-b, $p<0.001$), further confirming the efficient blocking of protein translation by cycloheximide in the Hpt cells. These data strongly suggest that the intracellular trafficking of WSSV via the endosomal system could be abolished by the lack of *Cq*VCP ATPase activity, which subsequently led to the efficient inhibition of delivery of the WSSV genome into the Hpt cell nucleus for successful viral replication. Most importantly, the high level of accumulated and

colocalized viral envelope protein VP28 and nucleocapsid protein VP664 within the Hpt cell nucleus area, as an indication of the newly assembled WSSV progeny virions at a relatively late infection stage 18 hpi, a time proven to be a stage suitable for assembly of the progeny virions in red claw crayfish Hpt cells *in vitro* (33), was found mostly in the nucleus of the control Hpt cells not treated with DBeQ as shown by immunostaining analysis (Fig. 3F, upper). In contrast, immunofluorescence signal of the progeny WSSV virions was found to be negligible in the nucleus of the Hpt cells lacking *Cq*VCP ATPase activity because of DBeQ exposure, but in which the rarely remaining WSSV components, such as VP28 and VP664, were present only in the cytoplasmic area of the Hpt cells and were likely to be the endocytic virions that had not been efficiently degraded due to the blocked viral fusion, which was necessary for efficient degradation. Similar results were also found in the Hpt cells examined by TEM during our experiments; that is, no progeny WSSV virion was found in the nuclei of the Hpt cells lacking *Cq*VCP ATPase activity because of DBeQ exposure, in contrast to the number in the control cells (Fig. 3F, lower). These findings strongly indicate that the endosomal delivery pathway of the endocytic WSSV virions, regulated by the *Cq*VCP ATPase activity inducing viral fusion with the endosome membrane, is extremely critical for the successful propagation of WSSV.

As NEDD8-activating enzyme might link the VCP pathway to cullin-ring ubiquitin ligases during substrate presentation (42) and the inhibition of proteasome activity blocks the release of mouse hepatitis virus from the endosome to the cytosol (43), we questioned whether the cullin-ring ubiquitin ligases-VCP pathway regulated the

endosomal trafficking of WSSV, considering that the cullin-ring ubiquitin ligases 3 functions upstream of VCP-segregase to regulate endosome maturation (44). The effect on WSSV infection was determined after inhibiting NEDD8-activating enzyme 1 activity with the inhibitor MLN4924 (45) or blocking proteasome activity with MG132. Meanwhile, the acidic endosomes labeled by LysoTracker clearly accumulated due to the blocking of NEDD8-activating enzyme 1 or proteasome activity (data not shown). However, WSSV fusion was not obviously affected by the disruption to the *Cq*VCP-proteasome pathway (data not shown), implying that maturation of the late endosomes is not needed for WSSV infection. In addition, the ubiquitin-proteasome degradation pathway, indicated by the higher presence of ubiquitin is unable to be degraded, was inhibited in the Hpt cells either by dysfunction of *Cq*VCP through DBeQ treatment or by blocked proteasome degradation through MG132 (data not shown). The ubiquitinated fragments caused by blocking of these two cellular processes exhibited differences. These data together implied that the *Cq*VCP-proteasome degradation pathway was not clearly associated with the endosomal trafficking of WSSV in the Hpt cells.

Autophagy was induced by aggregated WSSV virions in endosomes rendered dysfunctional by the lack of *Cq*VCP ATPase activity

As shown above, the WSSV virions were “trapped” within the dysfunctional endosomes caused by the lack of *Cq*VCP ATPase activity (Fig. 3C and Fig. 3D). The conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was significantly induced by the lack of *Cq*VCP activity via gene silencing (Fig. 4A), which has also been reported in

mammalian cells but without mechanistic details (41). In addition, the formation of *Cq*GABARAP puncta (Fig. 4B-a&b) as well as the conversion of *Cq*GABARAP-I to *Cq*GABARAP-II (Fig. 4B-c) were significantly increased in Hpt cells upon DBeQ treatment in a dose-dependent manner, suggesting that autophagic activity was strongly promoted by blocking *Cq*VCP ATPase activity in the Hpt cells. Importantly, the aggregated WSSV virions were clearly surrounded by *Cq*GABARAP puncta that were increased, in a DBeQ-dependent manner, in the Hpt cells (Fig. 4B-d). This finding led to a question regarding how *Cq*VCP activity was associated with autophagy, as *Cq*GABARAP-associated autophagy was involved in regulating WSSV infection, as we previously described (12). To characterize the proposed association between *Cq*VCP and *Cq*GABARAP, a colocalization assay with immunostaining was performed in the Hpt cells after blocking of *Cq*VCP ATPase activity with DBeQ and infection by WSSV. As shown in Fig. 4C-a (upper), the colocalization of *Cq*VCP and *Cq*GABARAP was dramatically increased in the dysfunctional endosomes in the Hpt cells because of the blocked *Cq*VCP ATPase activity; however, it was negligible in the control cells. In addition, the dysfunctional endosomes, as indicated by WGA staining, were surrounded by accumulated *Cq*GABARAP puncta (Fig. 4C-a, lower), which were also shown to surround the WSSV aggregates, as indicated by immunostaining against VP28 (Fig. 4C-b, upper). In particular, the WSSV virions were found to be accumulated in these dysfunctional endosomes because of the lack of *Cq*VCP ATPase activity, and the accumulated WSSV virions were surrounded by the accumulated *Cq*GABARAP puncta in the Hpt cells (Fig. 4C-b, lower). These results clearly

demonstrated that *Cq*GABARAP was recruited to dysfunctional endosomes containing aggregated WSSV virions because of the defect in *Cq*VCP ATPase activity, and it was likely degraded in the autophagosome-lysosome pathway after the endocytic virions were entrapped within these dysfunctional endosomes, in regarding to that the viral replication was significantly reduced by blocking *Cq*VCP ATPase activity with DBeQ as shown in Fig. 3B-b. However, it is unknown whether *Cq*VCP acts as an autophagic adaptor protein in the abovementioned process. During the recognition and sorting of autophagic substrates, the autophagy receptor links ubiquitinated substrates to autophagosome membranes via the LC3-interacting region (46). For instance, mammalian VCP has a classic LIR motif containing a consensus sequence of W/F/YXXL/I/V. By homologous analysis of *Cq*VCP, two LIRs were found in the CDC48-2 domain of *Cq*VCP (Fig. 4D-a). To determine whether *Cq*VCP binds to *Cq*GABARAP, the recombinant *Cq*VCP protein with a His-tag was prepared for a protein-binding assay with recombinant GST-*Cq*GABARAP protein *in vitro*; the pull-down assay showed that rHis-*Cq*VCP could bind to rGST-*Cq*GABARAP (Fig. 4D-b), suggesting a possible key role for the LIR motif in the *Cq*VCP interaction with *Cq*GABARAP, which might be critical for recruiting autophagosomes and, by binding to *Cq*GABARAP, inducing their fusion with endosomes containing the aggregated WSSV virions for the subsequent degradation of invading WSSV.

Dysfunctional endosomes containing WSSV aggregates, because of blocked *Cq*VCP ATPase activity, could trigger autophagy and recruit autophagosomes that fuse with these endosomes. To determine how autophagic activity is induced by the

dysfunction of *Cq*VCP in the Hpt cells (Fig. 4A and Fig. 4B), the ATP hydrolysis propagation of *Cq*VCP was hindered by maintaining D2 in an ADP-bound state with the chemical inhibitor NMS873, which has been proposed to bind to the inter-subunit signaling (ISS) motif of VCP (47). As speculated, the inhibitor NMS873 efficiently bound to recombinant *Cq*VCP, as determined by the biolayer interferometry assay, and the analysis of the steady state of recombinant His-*Cq*VCP with NMS873 showed a high affinity, with KD=8.7E-10 M (data not shown). Next, the ATP hydrolysis propagation of *Cq*VCP was hampered by NMS873 treatment of the Hpt cells for 2 h, followed by blocking of *Cq*VCP ATPase activity with DBeQ (41). In contrast to the significantly increased conversion of *Cq*GABARAP-I to *Cq*GABARAP-II found by blocking only ATPase activity with DBeQ, no clear difference in conversion level was found either in the cells in which D2 in *Cq*VCP was restricted in the ADP-bound state, followed by continual blocking of ATPase activity, or in the cells with in which only D2 in *Cq*VCP was restricted in the ADP-bound state (Fig. 4E-a). We then speculated that the reason for this phenomenon was that pre-binding NMS873 to the *Cq*VCP-ISS motif abolished the subsequent binding of DBeQ to the ATP-binding site in *Cq*VCP (40, 46), leading to the subsequent blockade of the autophagy induced by dysfunctional *Cq*VCP ATPase activity. Additionally, when endosomal V-ATPase activity was pre-inhibited by the specific chemical inhibitor bafilomycin-A1 for 2 h in the Hpt cells, followed by blocking of ATPase activity with DBeQ, no significant conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was found, compared to the conversion in the Hpt cells lacking only the *Cq*VCP ATPase activity upon DBeQ

544 treatment (Fig. 4E-b). Consistently, the formation of *Cq*GABARAP puncta induced
545 by the *Cq*VCP-DBeQ complex was significantly reduced by maintaining the *Cq*VCP
546 conformation with NMS873 or by inhibiting V-ATPase with bafilomycin-A1 ($p<0.01$)
547 (Fig. 4E-c), suggesting that the autophagy induced by dysfunctional *Cq*VCP activity
548 within endosomes was directly associated with V-ATPase activity in the endosomes.
549 Taken together, these data demonstrated that the endosomal trafficking of WSSV
550 could be efficiently disrupted by abrogating *Cq*VCP ATPase activity, at which time
551 autophagy was recruited to segregate and likely eliminate endocytic WSSV virions
552 within the dysfunctional endosomes via autophagic degradation.

553 **Autophagy played a defensive role but was not completely efficient in**
554 **attenuating WSSV infection**

555 As shown above in the current study, many WSSV aggregates within the
556 dysfunctional endosomes were suggested to be fused with autophagosomes (Fig. 4C).
557 However, it remains unclear whether the WSSV virions trapped within
558 autophagosomes are degraded by the autophagy pathway. To clarify this unknown
559 mechanism, induction of the autophagic activity against possibly accumulated
560 endocytosed WSSV virions was carried out by infecting Hpt cells with a relatively
561 high multiplicity of infection (MOI) of 10, *i.e.* leading to a speculated "overloading"
562 of intracellular virions incapable of proper trafficking, to mimic the possible effect on
563 WSSV trafficking caused by blocking of the endosomal delivery as identified in Fig.3.
564 We confirmed that, upon both protein expression level of *Cq*GABARAP and the
565 conversion of *Cq*GABARAP-I to *Cq*GABARAP-II, an autophagy marker protein,

566 was significantly increased by WSSV infection 6 hpi in the Hpt cells (Fig. 5A),
567 indicating that the autophagic activity was indeed increased during the early stage of
568 WSSV infection with a relatively high viral number, but only showing a slight
569 increase lacking statistic difference if using an MOI of 5 (data not shown). To further
570 determine the association between autophagic activity and WSSV infection,
571 autophagic activity was artificially promoted with a potent selective Akt 1/2 kinase
572 inhibitor (AKTi 1/2) to decrease the phosphorylation of AKT-pS473 in the Hpt cells,
573 because autophagy is centrally regulated by the classic PI3K-AKT/mTOR signaling
574 pathway (48). As shown in Fig. 5B, autophagic activity, indicated by both the
575 formation of *Cq*GABARAP puncta (Fig. 5B-a&b) and the conversion of
576 *Cq*GABARAP-I to *Cq*GABARAP-II (Fig. 5B-c), was clearly promoted in Hpt cells
577 by AKTi 1/2 in a dose-dependent manner. To further determine the effect of
578 autophagic activity on WSSV infection, autophagic activity was promoted with AKTi
579 1/2 in the Hpt cells before WSSV infection. As determined by the real-time dynamic
580 analysis, more endosomes labeled by WGA, containing DiD-WSSV virions, were
581 shown to accumulate and fuse with each other to form enlarged vesicles 3 hpi in live
582 Hpt cells with higher levels of induced autophagic activity (Fig. 5C and Supplemental
583 Video 4). Furthermore, when the autophagic activity was increased by AKTi 1/2 in
584 the Hpt cells, many more WSSV virions, as indicated by anti-VP28 immunostaining,
585 were found to be colocalized and accumulated in endosomes, as indicated by
586 anti-RabGEF1 immunostaining (Fig. 5D-a, upper panel), suggesting that more
587 endosomes containing WSSV virions accumulated and fused with each other to form

enlarged vesicles. In particular, these accumulated endosomes, as indicated by WGA staining, were also shown to be extensively colocalized with the puncta of the autophagosome marker protein *Cq*GABARAP, showing that the WSSV-containing endosomes were likely to be fused with autophagosomes, according to the uniformly dispersed fluorescence of the *Cq*GABARAP puncta and WGA dye in the Hpt cells with increased autophagic activity (Fig. 5D-a, lower). In addition, the accumulated WSSV aggregates, as indicated by the immunostaining on VP28, were obviously surrounded by *Cq*GABARAP puncta, as the fluorescence intensity of the colocalized *Cq*GABARAP and VP28 was significantly higher in the cells with the promoted autophagic activity, as shown in the histogram analysis results ($p<0.001$), strongly supporting the supposition that the aggregated WSSV virions are located within autophagosomes (Fig. 5D-b, upper). Most importantly, the fusion of the viral envelope with the endosomal membrane was profoundly blocked by increased autophagic activity, as most viral envelope proteins were shown to be extensively colocalized with the nucleocapsids at a level significantly different ($p<0.001$) in the Hpt cells with promoted autophagic activity (Fig. 5D-b, lower). Furthermore, the segregation of these intracellular WSSV virions by the enhanced autophagic activity, leading to the formation of more WSSV virion aggregates within autophagosomes, was also confirmed by TEM, in which the relative percentage of autophagosomes with the typical double-layer membranes containing more than 3 virions per autophagosome was much higher than that of autophagosomes containing less than 3 aggregated WSSV virions per autophagosome (Fig. 5D-c), indicating that the higher

610 autophagic activity may have increased the accumulation of WSSV virions into
611 autophagosomes. To further support this speculation, both the WSSV protein
612 component levels and the transcription levels of viral genes were determined for the
613 Hpt cells with induced autophagic activity. As shown in Fig. 5E-a, a WSSV protein
614 component, for example, the envelope protein VP28, was significantly reduced by the
615 increased autophagic activity 12 hpi in the Hpt cells, in which the protein translation
616 activity was pre-blocked by cycloheximide (Fig. 5E-a, $p<0.05$). In the control group
617 without cycloheximide treatment, the VP28 protein level was significantly lower in
618 the cells with higher autophagic activity than it was in the cells without AKTi
619 1/2-induced autophagic activity, suggesting that the higher autophagic activity led to
620 more efficient degradation of the invading WSSV. In addition, viral gene transcription,
621 such as that of the VP28 gene, exhibited a clear decrease 12 hpi (Fig. 5E-b, $p<0.01$),
622 which was presumed to be caused by the induced autophagic sorting and the
623 subsequent degradation of endocytic WSSV virions within autophagosomes, as shown
624 above (Fig. 5E-a). Consistently, the higher autophagic activity level led to much more
625 efficient inhibition of WSSV replication in the control group without cycloheximide
626 treatment (Fig. 5E-b). However, the higher autophagic activity induced by AKTi 1/2
627 neither led to the complete degradation of invading WSSV nor thoroughly blocked
628 viral replication at the relatively late infection stage, 12 hpi (Fig. 5E), implying that
629 some virions might escape autophagic elimination.

630 Interestingly, when autophagic activity was promoted by AKTi 1/2, the number of
631 accumulated acidic autophagosome-lysosome vesicles, indicated by the accumulated

632 fluorescent signal of the Lysotracker staining, were greatly increased in the Hpt cells,
633 in which both the fluorescence intensity of the Lysotracker dye and the relative size of
634 the accumulated vesicles were significantly increased (Fig. 5F, upper, $p<0.01$). In
635 addition, the presence of proteinase cathepsin L, an indicator of the degradation
636 within autophagosomes (49), was dramatically increased in the Hpt cells with higher
637 autophagic activity, in which the relative activity of cathepsin L was significantly
638 higher than that of control cells (Fig. 5F, $p<0.01$, lower). This result directly
639 confirmed that the autophagic degradation activity was likely to be increased by the
640 acidic conditions promoted by the AKTi 1/2 treatment in the Hpt cells. Taken together,
641 these findings strongly demonstrated that, in contrast to the endosomal trafficking
642 system, high autophagic activity was capable of enhancing the segregation of
643 endosomes containing WSSV virions and their fusion with autophagosomes, leading
644 to the subsequent degradation of WSSV via the autophagosome-lysosome pathway.

645 **WSSV escaped autophagic elimination by entering the endosome delivery system**
646 **facilitated by *Cq*VCP**

647 As described above, both the endosomal delivery pathway and the autophagic
648 degradation pathway were triggered during WSSV infection. A key question involves
649 the fate of WSSV trafficking, *i.e.*, does it follow the endosome delivery pathway to
650 release the viral genome into the cell nucleus, or does it follow the autophagy
651 pathway to be degraded? To determine WSSV fate, the intracellular trafficking of
652 WSSV was compared in Hpt cells with dysfunctional endosomes lacking *Cq*VCP
653 activity and cells with increased autophagic activity. As shown by the fluorescent

signal of WSSV, indicated by the immunostained envelope protein VP28, all artificial treatments, *i.e.*, the promoted autophagic activity induced by AKTi 1/2 action against AKT, the dysfunctional endosomes that lacked *Cq*VCP ATPase activity because of DBeQ treatment, and the promoted autophagic activity and the dysfunctional endosomes caused by treatment of both inhibitors together, resulted in significantly increased WSSV accumulation and aggregation in the cytoplasm (Fig. 6A-a, upper; Fig. 6A-b, $p<0.01$ or 0.001), as well as the profound inhibition of viral fusion with endosome membrane, as indirectly indicated by the extensive colocalization of VP28 and VP664 (Fig. 6A-a, lower; Fig. 6A-c, $p<0.01$ or 0.001), in the Hpt cells 4 hpi, compared to these measures in the negative control Hpt cells. In particular, endosome dysfunction in the Hpt cells resulted in significantly greater WSSV aggregation and inhibition of viral fusion with the endosome membrane than was observed in cells with increased autophagic activity (Fig. 6A-b&c, $p<0.01$). In addition, no significant difference was observed between the Hpt cells with the dysfunctional endosomes plus the increased autophagic activity and the cells with only dysfunctional endosomes in terms of WSSV aggregation and viral fusion with the endosomal membrane (Fig. 6A-b&c, $p>0.05$). However, this was not the case in the cells with only increased autophagic activity, which exhibited significantly lower viral aggregation and reduced inhibition of fusion compared to the cells with both dysfunctional endosomes and promoted autophagic activity (Fig. 6A-b&c, $p<0.01$). These findings together demonstrated that most of the intracellular WSSV virions were directed to the endosomal trafficking pathway; *i.e.*, they escaped the autophagy degradation pathway.

676 Consequently, disruption of endosomal function, *i.e.*, by inhibiting *Cq*VCP ATPase
677 activity resulted in a significantly stronger blocking effect on the proper trafficking of
678 WSSV than was achieved by increasing autophagic activity, which then led to much
679 more efficient inhibition of viral replication in the Hpt cells at a very late infection
680 stage, 24 hpi (Fig. 6A-d). Accordingly, no significant difference was found in viral
681 transcription in the Hpt cells with dysfunctional endosomes and those Hpt cells with
682 both dysfunctional endosomes and increased autophagic activity, suggesting that
683 WSSV could take advantage of the endosomal delivery system, directly regulated by
684 *Cq*VCP ATPase activity, to maintain proper intracellular transport of the WSSV
685 genome to the Hpt cell nucleus for viral replication, and it was presumed that most of
686 the intracellular virions successfully escaped autophagic elimination.

687 As shown above, more endosomes containing the endocytic virions accumulated
688 and fused with each other to form endosomes with enlarged sizes, as determined by
689 live-cell imaging of the cytosolic trafficking of WSSV in the Hpt cells with promoted
690 autophagic activity (Fig. 5C), which was followed by increased fusion with
691 autophagosomes (Fig. 5D). Another question was directed to determining whether
692 more WSSV virions within these endocytic vesicles would enter the autophagy
693 pathway, leading to the complete blocking of viral infection, if the pathway by which
694 endocytic virions enter endosomes was blocked. In addition, is *Cq*VCP involved in
695 the regulation of WSSV sorting as is the case in mammals, for which VCP has been
696 reported to be critical for the homotypic fusion of Golgi fragments in HeLa cells (50)?
697 To address these questions, the tentative homotypic fusion between WSSV-containing

endocytic vesicles and endosomes was pre-abolished in Hpt cells by an NMS873 inhibitor, which was capable of restricting D2 in VCP in the ADP-bound state and then disrupting the endomembrane fusion mediated by the VCP/p97-p47 membrane fusion machinery (51). Intriguingly, the viral fusion of endocytic WSSV virions was severely inhibited, as indicated by the significant presence of fluorescent VP28 in the cytoplasm of the Hpt cells upon NMS873-induced blockage of the fusion between the WSSV-containing endocytic vesicles and endosomal membranes; *i.e.*, disruption of viral trafficking via the endosomal delivery system at an early infection stage, 4 hpi, had an effect on endocytosed virions (Fig. 6B-a, upper, $p<0.01$). As mentioned in Fig. 2A and Fig. 2D, the endocytic WSSV virions accumulated and detained in the endosomes in the alkalized Hpt cells with chloroquine, but it was unclear whether the proposed homotypic fusion between the endocytic vesicles containing WSSV and the endosomes occurred after alkalization in the Hpt cells. To address this speculation, the proposed homotypic fusion was pre-abolished by NMS873 in the Hpt cells, which were then subjected to alkalization with chloroquine. Similar to the results in Fig. 2A&D above, homotypic fusion between the WSSV-containing endocytic vesicles and endosomes occurred as usual, even under alkalization by chloroquine, in the Hpt cells; that is, the accumulated virions were aggregated within the alkalized endosomes, as indicated by the fluorescence intensity of VP28 colocalized with RabGEF1, but without viral envelope fusion to the endosomal membrane due to the disrupted acidic condition that was a prerequisite for viral envelope fusion (Fig. 6B-a, the lower number of control cells). However, the accumulation or aggregation of the endocytic

720 WSSV virions was significantly reduced in the Hpt cells in which the
721 *Cq*VCP-mediated homotypic fusion was pre-abolished by NMS873, followed by
722 alkalization with chloroquine (Fig. 6B-a, the lower, $p<0.01$). Interestingly, when the
723 homotypic fusion described above was abolished with NMS873, the conversion of
724 *Cq*GABARAP-I to *Cq*GABARAP-II, indicating increased phagocytic activity, was
725 significantly increased by WSSV infection in a viral dose-dependent manner in the
726 Hpt cells (Fig. 6B-b), suggesting that more virions were likely to be directed to the
727 autophagosome once the endosomal delivery pathway was abolished by dysfunctional
728 *Cq*VCP activity, as speculated above. In summary, in addition to its role in
729 maintaining the endosome acidification shown above (Fig. 3), *Cq*VCP was likely to
730 facilitate homotypic fusion between endocytic vesicles and endosomal vesicles in Hpt
731 cells, in which homotypic fusion was speculated to be efficiently blocked by
732 maintaining D2 in *Cq*VCP in an ADP-bound state with NMS873.

733 As described above, more endocytic WSSV virions were likely to be directed to
734 the autophagy pathway after the homotypic fusion between endocytic vesicles and
735 endosomes were blocked (Fig. 6B-b), but it was unclear whether blocking homotypic
736 fusion mediated by *Cq*VCP affected the autophagic activity. To address this
737 speculation, Hpt cells were pretreated with NMS873 for 2 h to block homotypic
738 fusion, followed by AKTi 1/2 treatment to increase autophagic activity. Similar to the
739 negative control cells without inhibitor treatment, no obvious conversion of
740 *Cq*GABARAP-I to CqGABARAP-II was found in the Hpt cells after restricting D2 in
741 *Cq*VCP in an ADP-bound state, compared to that in the cells with higher autophagic

activity (Fig. 6C-a). Particularly, when the *Cq*VCP-ADP state was preemptively maintained by NMS873 in the Hpt cells, and then, when the autophagic activity was stimulated with AKTi 1/2, no significant difference was found in the conversion of *Cq*GABARAP-I to *Cq*GABARAP-II in these cells, compared to that in the cells with the autophagic activity promoted by AKTi 1/2 alone, which suggested that blocking homotypic fusion by restricting the *Cq*VCP-ADP state did not affect the conversion of *Cq*GABARAP-I to *Cq*GABARAP-II, *i.e.*, the increased autophagic activity induced by AKTi 1/2. Interestingly, both *Cq*GABARAP punctate formation and the presence of acidic vesicles were clearly reduced by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873, in comparison to the negative control cells without inhibitor treatment, and the punctate formation and number of acidic vesicles were also significantly lower than they were in the positive control cells with the higher autophagic activity, as induced by AKTi 1/2 (Fig. 6C-b). In addition, almost no clear formation of *Cq*GABARAP puncta was evident in the Hpt cells with *Cq*VCP activity pre-abolished with NMS873 treatment for 2 h, followed by increased autophagic activity with AKTi 1/2 treatment, and the presence of acidic vesicles was also clearly decreased in the NMS873 treated cells compared to that of positive control cells with autophagic activity promoted by AKTi 1/2 alone. This finding strongly demonstrated that the formation of *Cq*GABARAP puncta was dramatically disrupted by restricting D2 in *Cq*VCP in an ADP-bound state, which also indirectly showed that endomembrane fusion, such as autophagosome accumulation, was efficiently blocked by abolishing *Cq*VCP activity through maintenance of the ADP-bound state of D2 in

764 *Cq*VCP. To further reveal whether this homotypic fusion indeed affected the direction
765 of WSSV into autophagosomes, as speculated above (Fig. 6B-b), homotypic fusion
766 was pre-abolished with NMS873, and the autophagic activity was increased with
767 AKTi 1/2 in the Hpt cells, which were subsequently infected with WSSV. Importantly,
768 fewer WSSV virions were shown to accumulate within autophagosomes in the Hpt
769 cells when homotypic fusion between endocytic vesicles containing WSSV and
770 endosomes was pre-abolished by restricting D2 in *Cq*VCP in an ADP-bound state
771 through NMS873 treatment. In particular, more WSSV virions were dispersed in the
772 endocytic vesicles without obvious accumulation in the cytoplasm, indicating that
773 homotypic fusion was important for further delivery of the endocytic virions into or
774 fusion with the organelles targeted for viral trafficking, including both endosomes and
775 autophagosomes. In contrast, a significant accumulation of WSSV virions in
776 autophagosomes was present in the control cells without NMS873 treatment (Fig.
777 6C-c, $p<0.01$). These data suggested that the homotypic fusion mediated by *Cq*VCP
778 was important for directing the endocytic WSSV into autophagosomes. Consistently,
779 WSSV replication was also significantly inhibited by blocked homotypic fusion of
780 endocytic vesicles containing WSSV with endosomes as mediated by *Cq*VCP with
781 NMS873 treatment (Fig. 6D, left, $p<0.001$), likely leading to the subsequent blockade
782 of delivery of the WSSV genome into the Hpt cell nucleus. In addition, fewer viral
783 components, such as VP28, were present in the Hpt cells lacking *Cq*VCP activity
784 because of NMS873 treatment at a very late infection stage, 24 hpi, indicating that a
785 negligible amount of viral genome was delivered into the cell nucleus for viral

replication and the subsequent synthesis of viral proteins, compared with the amount delivered in the control cells (Fig. 6D, right, $p<0.001$). Most importantly, the assembled WSSV virion progeny, as indicated by the accumulated fluorescence of colocalized VP28 and VP664, was almost solely present in the control cells without inhibited homotypic fusion by NMS873 at a late stage of WSSV infection, 18 hpi, as shown by the immunostaining analysis results (Fig. 6E, upper). In addition, the relative percentage of Hpt cells with progeny virions was significantly lower in the cells lacking *Cq*VCP activity due to NMS873 inhibition than it was in the negative control cells without inhibitor treatment (Fig. 6E, right, $p<0.001$), in which negligible fluorescent signals from the progeny WSSV virions was observed in the nucleus of the Hpt cells lacking *Cq*VCP activity because of NMS873 exposure. Furthermore, the few remaining WSSV components, such as VP28 and VP664, present only in the cytoplasmic area of Hpt cells, were likely to be the endocytic virions that had not been efficiently degraded due to the blocked homotypic fusion and hindered viral fusion necessary for subsequent efficient degradation within the time selected. Similar results were also found in the Hpt cells examined by TEM during our experiments, with negligible progeny of WSSV virions found in the nucleus of the Hpt cells lacking homotypic fusion activity, mediated by blocking *Cq*VCP activity with NMS873, compared to that of control cells (Fig. 6E, lower). These findings strongly indicated that the endosomal delivery pathway of the endocytic WSSV virions, regulated by *Cq*VCP activity during both endocytic vesicle fusion with endosomes and viral fusion with the endosome membrane to avoid autophagic clearance, was

808 extremely important for the successful propagation of WSSV.

809

810 **DISCUSSION**

811 Previous studies found that multiple endocytic routes were used by WSSV for entry
812 into host cells, but the subsequent intracellular trafficking of WSSV remained largely
813 unknown. To better understand WSSV pathogenesis, as well as the antiviral response
814 in crustaceans, we established live-cell imaging of WSSV infection, for the first time,
815 in the current study, which may provide important clues for the intracellular
816 trafficking of WSSV. Endocytosis and the subsequent endosomal trafficking provide
817 an effective way by which many enveloped DNA viruses deliver their genetic material
818 into the host cell nucleus for successful infection. By using the established live-cell
819 imaging observation on the dynamic trafficking of intracellular WSSV labeled with
820 fluorescent dye together with immunostaining examination, we found that the
821 internalized endocytic WSSV virions were transported into the endosomal vesicles,
822 where viral envelope fusion with the endosomal membranes indeed occurred under
823 acidic conditions within the endosomal compartment. In general, the fusion of the
824 enveloped DNA virus with endosomes and the subsequent penetration of the viral
825 nucleocapsid enclosing the viral genome into the cytosol occur sequentially during
826 viral infection via the endocytic pathway, in which the acidity of the late endosome is
827 critical for viral fusion. However, WSSV envelope fusion with the endosomal
828 membrane can be achieved in early endosomes but is not necessary because, upon
829 conversion of early endosomes to late endosomes, the acidification, likely at pH 6.5 in
830 early endosomes, is proposed to be sufficient for WSSV fusion. It is likely that the

831 WSSV fusion is similar to that of the insect baculovirus *Ac*MNPV, in which envelopes
832 fused with the membrane of early endosomes after cell entry through mainly
833 clathrin-mediated endocytosis, allowing for nucleocapsid release into the cytoplasm
834 (52). In contrast, the deencapsidation of other enveloped DNA viruses, such as the
835 ASFV, in which both the outer envelope rupture and the inner envelope fusion occur
836 at the acidic pH of late endosomes, the inner viral membrane was exposed and fused
837 with the limiting endosomal membrane to release the viral core into the cytosol in a
838 process that was dependent on acidic pH and the inner envelope viral protein pE248R
839 (10). Moreover, the mechanism in which the fusion machinery and proteins are
840 involved in WSSV infection has not been identified. In particular, how viral fusion is
841 initiated by binding of the WSSV envelope with the endosomal membrane is unclear.
842 Therefore, a better understanding of these processes, including the initiation of viral
843 fusion as well as the penetration of the nucleocapsid enclosing the viral genome into
844 the cytosol, will definitely provide a novel promising antiviral strategy against
845 WSSV.

846 Notably, WSSV envelope components, such as VP28 protein, are likely to be
847 efficiently degraded within 2 hpi after viral fusion with the endosomal membrane in
848 the Hpt cells. In contrast, most of the viral VP28 protein was present in the Hpt cell
849 cytoplasm for a long time, until 6 hpi, even lasting up to 18 hpi in the late infection
850 stage, after viral fusion was blocked as mediated by both a lack of *Cq*VCP activity
851 (either by gene silencing or by pharmacological inhibitors) and alkalization treatment
852 during our experiments. We then proposed that *Cq*VCP likely functioned in

maintaining the endosomal acidity necessary for successful fusion of the viral envelope with the endosomal membrane, which was a prerequisite for the subsequent efficient degradation of the viral components, as well as for the penetration of the WSSV nucleocapsid enclosing the viral genome into the cytosol, followed by release of the viral genome into the host cell nucleus for replication. Furthermore, whether the detached viral envelope proteins are transported to the endolysosome for degradation or by other protein degradation pathways, such as the proteasome pathway, has not been determined. Therefore, a better understanding of the processes related to *Cq*VCP will definitely contribute to the elucidation of WSSV pathogenesis since the propagation of WSSV could be almost completely abolished by blocking *Cq*VCP ATPase activity with DBeQ (Fig. 3F).

Intriguingly, we found that *Cq*VCP activity was also extremely critical for maintaining homotypic fusion between endocytic vesicles containing WSSV and endosomes, and was likely also important for the homeostasis of endosomes, at least in terms of their acidification, which ensured the proper intracellular trafficking of WSSV and the subsequent delivery of the viral genome into the host cell nucleus. VCP protein is mainly distributed in the cell cytoplasm, endosomes and nucleus and assembles as a hexameric complex, formed by six identical protomers, for biological functionality (47). In addition to the motifs such as CDC48-2 and LIR in *Cq*VCP that are conserved among other VCPs such as *Hs*VCP, we used a biolayer interferometry assay to identify that both DBeQ and NMS873 inhibitors could bind to *Cq*VCP, confirming that *Cq*VCP likely has conserved activities in crustacean similar to those

of humans, as mentioned above. Importantly, dysfunction of the *Cq*VCP activity induced with either DBeQ or NMS873, respectively, led to both significant inhibition of WSSV replication and profound blocking of the successful assemble of the progeny virions in the host cells lacking *Cq*VCP activity and that acquire a different phenotype during viral infection. For example, most of the endocytic WSSV virions were detained in the endocytic vesicles after their detachment from the cell membrane when the *Cq*VCP activity was disrupted by NMS873, suggesting that the homotypic fusion between the endocytic vesicles containing WSSV and the endosomes likely needed *Cq*VCP activity, presumably via its ATP hydrolysis propagation and active D2 in the ADP-bound state, as proposed above. In contrast, most of the endocytic WSSV virions accumulated in the endosomes without viral envelope fusion when the *Cq*VCP ATPase activity was disrupted by DBeQ pretreatment, indicating that *Cq*VCP ATPase activity was also critical for maintaining the acidic pH environment within endosomes, which was a prerequisite for viral envelope fusion with endosomal membranes. In contrast, no clear inhibition of WSSV fusion was observed by YM-201636 treatment by blocking the conversion of the early endosomes into late endosomes in Hpt cells, and inhibition of V-ATPase activity with bafilomycin-A1 strongly inhibited WSSV fusion with endosomal membranes, which was accompanied by significantly inhibited WSSV replication due to the decreased acidity within the endosomes (Fig. 2F). Therefore, the *Cq*VCP ATPase activity-regulated acidification within endosomes is likely associated with V-ATPase activity, but the mechanism still needs more investigation. These results demonstrate that *Cq*VCP functions, at least with dual roles,

in both homotypic fusion between WSSV-containing endocytic vesicles and endosomes and viral envelope fusion with endosomal membranes. However, the questions remain to be further addressed; for example, how is *Cq*VCP activity regulated by WSSV, in terms of both viral components and viral replication considering that *Cq*VCP gene transcription as well as protein synthesis is responsive to WSSV infection from the very early infection stage of 1 hpi to the relatively late infection stage of 12 hpi in the Hpt tissue *in vivo* (Fig. 3A)? In particular, the *Cq*VCP protein also accumulated and colocalized with WSSV aggregates in the dysfunctional endosome caused by blocking *Cq*VCP ATPase activity (Fig. 3D). In addition, whether more *Cq*VCP is recruited to endosomes after WSSV infection, which might also be involved in the degradation of WSSV protein components, including both envelope proteins and nucleocapsid proteins, is worthy of further investigation because VCP also controls protein homeostasis by acting as a molecular segregase that extracts specific ubiquitin-modified client proteins and delivers them to the proteasome for degradation (40).

Autophagy is a conserved cellular process that has been implicated in the detection and elimination of invading intracellular pathogens, such as viruses and dysfunctional organelles, to maintain cellular homeostasis. In the current study, we found that autophagy played an antiviral role against WSSV infection in red claw crayfish Hpt cells, in which the increased autophagic activity could significantly reduce viral replication by the isolation and aggregation of endocytic WSSV, leading to significant inhibition of viral fusion and uncoating that eventually reduced viral replication.

919 Hence, the antiviral role of autophagy suggests that selective breeding of animals with
920 high autophagic activity, to some extent, can be a feasible strategy to improve
921 anti-WSSV disease in crustaceans. Unfortunately, WSSV replication could not be
922 completely inhibited by increasing only autophagic activity, strongly suggesting that
923 WSSV was likely to escape autophagic degradation or to hijack the autophagy
924 pathway for infection, but the molecular details are lacking. In addition, it is also not
925 clear whether WSSV components, such as envelope proteins, might "manipulate" the
926 cellular response to facilitate the intracellular trafficking of WSSV for infection, as
927 more than 30% (Fig. 6A) of intracellular WSSV could escape autophagic degradation
928 even when autophagic activity was promoted; this possibility is worthy of further
929 investigation. Importantly, we showed that the endocytic WSSV virions had two
930 destinies in Hpt cells: trafficking via the endosomal system to successful propagation
931 or directed into the autophagy pathway to be degraded. Notably, most of the
932 endocytic WSSV virions were directed into the endosomal delivery pathway
933 compared to the number of virions directed into the autophagy pathway. In particular,
934 both viral replication and the assembly of progeny virions could be almost completely
935 inhibited by dysfunctional endosomes by disrupted *Cq*VCP activity, in which fusion
936 events, including both homotypic fusion between WSSV-containing endocytic
937 vesicles and endosomes and viral fusion between the viral envelope and endosome
938 membranes, were completely blocked. These blockades subsequently resulted in the
939 efficient inhibition of penetration, as well as the detainment of WSSV in the cytosol,
940 which abolished its ability to deliver the viral genome into the host cell nucleus. More

941 importantly, the autophagosome was then recruited, mediated by *Cq*GABARAP,
942 likely via its binding to the LIR domain of *Cq*VCP, to subsequently degrade these
943 aggregated virions. On the other hand, dysfunction of *Cq*VCP activity, either by gene
944 silencing or by the chemical inhibitor DBeQ against *Cq*VCP ATPase activity, but not
945 by restricting the *Cq*VCP-ADP state with NMS873, could clearly induce autophagic
946 activity. It has been reported that the endosomal sorting complexes required for
947 transport-III CHMP2B mutant could deposit LC3-associated neurodegeneration, and
948 gene knockdown of TSG101 and Vps24 induced the intracellular segregation of p62
949 (53, 54), suggesting that the autophagic machinery was recruited for the elimination
950 of injured endosomes becoming dysfunctional. Additionally, VCP and its cofactors,
951 such as UBXD1 and PLAA, could cooperate with autophagy in the clearance of
952 damaged endolysosomes (55). Therefore, according to our findings described above,
953 it is possible that the *Cq*VCP conformation change caused by DBeQ binding might
954 facilitate or even strongly promote the *Cq*VCP binding motif, such as LIR, exposure
955 to *Cq*GABARAP; however, this was not the case for NMS873 binding. This
956 speculation might explain why endosomes are associated with *Cq*VCP activity; *i.e.*,
957 the ATPase activity leading to dysfunctional endosomes, when lacking, triggered
958 *Cq*GABARAP to initiate autophagy, followed by the formation of autophagosomes to
959 fuse with these dysfunctional endosomes, enclosing the aggregated WSSV virions, for
960 subsequent clearance via autophagosome-lysozyme degradation. However, how
961 endocytic WSSV virions manage successful delivery via endosomes and escape
962 canonical autophagic recognition and sorting is unclear. In addition, the autophagy

induced by dysfunction of *Cq*VCP ATPase activity within endosomes was presumed to be directly associated with V-ATPase activity (Fig. 4E), suggesting a tentative association between *Cq*VCP and V-ATPase activity in a context that triggers autophagy. Hence, the key molecules, such as *Cq*VCP or other tentative factors involved in this process, and the tentative association between *Cq*VCP and V-ATPase activity, that trigger autophagy are worthy of further mechanistic study to reveal the possible novel antiviral innate immune response, especially considering that V-ATPase-stimulated endosomal acidification is required for group A rotavirus uncoating (56). Additionally, as most of the endocytic WSSV virions were sorted into endosomes with subsequent delivery for viral infection, it is reasonable to speculate that WSSV components, particularly envelope proteins, may also "manipulate" endosomes or putative key molecules such as *Cq*VCP to avoid the recruitment of *Cq*GABARAP or the initiation of autophagy for successful escape from autophagic degradation. Further addressing this speculation will also be very important for the elucidation of WSSV escape from innate immune clearance and, certainly, antiviral design. Taken together, as briefly summarized in Fig. 7, our findings imply that *Cq*VCP-associated endosome activity can be taken as a promising marker for anti-WSSV breeding selection or novel target design against WSSV disease.

MATERIALS AND METHODS

Animal, Hpt cell culture and virus

Healthy red claw crayfish *Cherax quadricarinatus* was purchased from Yuansentai Agricultural Science and Technology Co., Ltd of Zhangzhou, Fujian, China, which

were maintained in aerated tanks at 25 °C. Only inter-molting male crayfish were used for experiments. Crayfish were determined to be WSSV-free by detection of viral VP28 gene with PCR before experiments. Hpt cells were prepared from Hpt of red claw crayfish and cultured as described by Söderhäll et al. (57). WSSV stock was amplified in swamp crayfish *Procambarus clarkii*., and prepared virus was quantified by absolute quantification with PCR (58) and then stored in -80°C for later use. For WSSV infection in Hpt cells, different MOI was used in Hpt cells unless otherwise stated, including 2 for all viral gene transcription detection using quantitative real-time PCR (qRT-PCR), 5 for all detection of the intracellular WSSV within a relatively early infection stage 12 hpi using Western blotting (except the protein synthesis for viral assembly using Western blotting or confocal microscopy at a very late infection stage 24 hpi), 10 for induction of the autophagy by overloading of accumulated virions in the cytoplasm, 80 for confocal fluorescence microscopy analysis or immunofluorescence assay, and 1,000 for TEM analysis. The unattached WSSV virons in the cell medium were removed after 30 min incubation on the ice followed by washing the cells twice with L-15 medium and replacing with fresh cell culture medium, if without other specification, and then transferred to 26 °C incubation for infection, unless otherwise stated. For viral challenge *in vivo*, red claw crayfish (about 15 ± 3 g body weight) was infected with 100 copy of WSSV in crayfish saline buffer via injection of the fourth walking leg followed by collection of the Hpt tissue 1, 6, and 12 hpi for RNA isolation with cDNA synthesis, or for protein extraction with Western blotting assay against CqVCP.

1008 **Reagents and antibodies**

1009 Lipophilic fluorescent dye DiD (1, 1'-Dioctadecyl-3,3,3',3'-Tetramethylindodi-
1010 carbocyanine, Life Technologies, USA) or DiO (3,3'-Dioctadecyloxacarbocyanine
1011 Perchlorate, Beyotime) binding to viral envelope proteins was used to label WSSV
1012 envelope as previously described (12, 34). Copy of WSSV or fluorescent dye labeled
1013 WSSV was quantified by absolute PCR (58). Akt inhibitor (Akti 1/2) against AKT
1014 was purchased from the ABcam (ab142088, USA) for induction of autophagy.
1015 Chloroquine diphosphate salt (Sigma, C6628, USA) and ammonium chloride (NH_4Cl,
1016 Beyotime, China) were taken as alkalizer agents to increase intracellular pH of Hpt
1017 cells. Bafilomycin A1 (HY-100558) to inhibit V-ATPase activity was bought from
1018 MedChem Express (USA). VCP inhibitor, DBeQ (S7199) and NMS873 (S7285) were
1019 purchased from Selleck (USA). PIKfyve inhibitor YM-201636 (HY-13228) was
1020 bought from MedChem Express (USA). Mouse anti-VP28 monoclonal antibody was
1021 kindly provided by Prof. Feng Yang (Third Institute of Oceanography, Ministry of
1022 Natural Resources, Xiamen, Fujian, China). Rabbit multiple-clonal anti-VP664
1023 antibody was kindly provided by Prof. Chu-fang Lo (National Cheng Kung University,
1024 Taiwan, China). Rabbit anti-GABARAP monoclonal antibody (ab109364) was
1025 purchased from ABcam (USA); Mouse anti-Actin monoclonal antibody was
1026 purchased from TRANS (China); Rabbit anti-RabGEF1 polyclonal antibody was
1027 purchased from ABclonal (USA); Rabbit anti-VCP polyclonal antibody (GTX124821)
1028 was purchased from Genetex (USA) for colocalization with VP28 unless othervise
1029 specified; Mouse anti-VCP monoclonal antibody (sc-136273) was purchased from

Santa cruz (USA) for colocalization with RabGEF1 and CqGABARAP, and Western blotting analysis unless otherwise specified; Dylight 488 labeled donkey anti-rabbit IgG antibody, Dylight 594 labeled Goat anti-rabbit IgG antibody, and Dylight 649 labeled goat anti-mouse IgG antibody were purchased from EarthOx Life Sciences (USA).

qRT-PCR analysis

Total RNA from above Hpt cell cultures was extracted using Trizol reagent (Roche, Mannheim, Germany). Complementary DNA synthesized with SMARTer^{TM} RACE cDNA Amplification kit (Clontech, Madison, Wisconsin, USA) was prepared for qRT-PCR expression analysis with PrimeScript^{TM} RT Reagent Kit (TaKaRa, Japan) according to manufacturer's instructions. Gene expression of *CqVCP* and a viral envelope gene *VP28* of WSSV were analyzed in Hpt cell samples with or without WSSV infection as indicated accordingly. PCR primers for *CqVCP*, *VP28* and *16S* RNA were shown in Table 1.

Inhibition or induction on cellular activity in Hpt cell cultures by pharmacological chemicals

Crayfish Hpt cells were cultured in 24-well plates (5×10^5 cells/500 μL) or 96-well plates (10^5 cells/100 μL) at 20°C as aforementioned, which were treated by the pharmacological chemicals with indicated exposure time accordingly and replaced with fresh cell culture medium for subsequent viral infection or examination. Alkalization was induced by chloroquine (10 μM) or NH_4Cl (1 mM) for 1 hour in Hpt cells. ATPase activity of *Cq*VCP was blocked by DBeQ (10 μM) unless otherwise

stated in Hpt cell cultures. For WSSV infection in Hpt cells with blocked *Cq*VCP activity, the cell cultures were pre-exposed to DBeQ for 4 h followed by infection with WSSV or fluorescent dye labeled virions including DiD-WSSV or DiO-WSSV. To induce autophagic activity in Hpt cells, cell cultures were pre-exposed to AKTi 1/2 (20 μM) for 6 h followed by examination or WSSV infection. To determine WSSV infection by live-cell imaging in real-time, Hpt cell cultures were infected by DiD-WSSV or DiO-WSSV, respectively, and the image was taken from 2 hpi. Acidic vesicles were stained with LysoTracker™ Red DND-99 (L7528, ThermoFisher, USA) (59). V-ATPase of endosomes was blocked by bafilomycin-A1 (10 μM) in Hpt cell cultures for 2 h followed by WSSV infection. In addition, DBeQ and NMS873 (10 μM) were employed to disrupt endosome activity via blocking *Cq*VCP activity, respectively. YM201636 (5 μM) was employed to disrupt endosome activity. Intracellular localization of WSSV was examined by LSM 780 confocal fluorescence microscope (Zeiss, Germany) or TEM (JEM2100HC) as described below. To block protein translation activity in Hpt cells, the cell cultures were exposed to cycloheximide (10 μg/mL, Sigma) for 2 h followed by WSSV infection or other chemicals exposure as indicated. No significant changes in cell viability were observed in the Hpt cells treated by those pharmacological chemicals with working concentrations used in this study when compared to those of the control cells (data not shown) unless otherwise stated. Effect on WSSV infection was determined with Western blotting on WSSV components or qRT-PCR on WSSV gene expression. Effect on WSSV infection by autophagy activity was determined by detecting

1074 degradation of viral VP28 protein with Western blotting as well as gene transcription
1075 of *VP28* with qRT-PCR.

1076 **Live-cell imaging on intracellular trafficking of WSSV**

1077 Intracellular acidic organelles were labeled by fluorescent dye LysoTracker™ Red
1078 DND-99 in live Hpt cells. Wheat germ agglutinin (Alexa Fluor® 488 WGA, Thermo
1079 Fisher, USA) was used to track endocytosis pathway, which binds to sialic acid from
1080 glycoprotein on cell membrane then accompanied with the following endocytic
1081 internalization and delivery into endosomes (37). Intracellular trafficking of live
1082 WSSV in live Hpt cells was monitored by confocal fluorescence microscope. Hpt
1083 cells were cultured in 96-well black plates (Cellvis, USA) for confocal imaging at a
1084 density of 10^5 cells/well at 20 °C. Fluorescent dye labeled WSSV, DiD-WSSV or
1085 DiO-WSSV, was innoculated to Hpt cell cultures and incubated for additional 30 min
1086 at 4 °C to allow viral attachment. Unattached virions were eliminated by removing
1087 medium and washing the cells twice with L-15 medium followed by addition of fresh
1088 cell culture medium. Synchronous entry was initiated by shifting Hpt cell culture to
1089 26 °C rapidly. Continuous dynamic images were recorded in real-time by laser
1090 confocal microscope.

1091 **Immunofluorescence assay**

1092 For immunofluorescence analysis of WSSV trafficking by confocal microscopy,
1093 double immunofluorescent staining was carried out in WSSV-infected Hpt cells.
1094 Briefly, Hpt cells were cultured on coverslips in a 96-well plate followed by WSSV
1095 infection for 4 hours at 26 °C. Cells on coverslip were fixed with 4%

paraformaldehyde for 30 min followed by permeabilization with 0.1% Triton X-100 for 30 min. After blocking with 5% goat serum for 1 h, Hpt cells were washed with PBS and incubated with primary antibody against VP28, VP664, *Cq*VCP, RabGEF1 or *Cq*GABARAP, respectively, diluted in 0.1% goat serum overnight at 4 °C. After washing three times with PBS, Alexa Fluor 488-conjugated goat anti-mouse IgG or Alexa Fluor 633-conjugated goat anti-rabbit IgG (1:200) was added and incubated for 2 h at room temperature. Hpt cell nucleus was stained with DAPI, and cell imaging was taken by confocal microscope.

TEM analysis

Hpt cells were seeded in 6-cm disks and pretreated by different pharmacological inhibitors as mentioned above accordingly followed by WSSV infection for 4 h unless otherwise stated for TEM analysis. Cell samples were harvested using a scraper, and centrifuged at 500×g for 3 min. Cell pellets were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde at 4 °C for 2 h. Fixed cells were dehydrated through an ethanol series, resin-embedded and sectioned with a microtome (Leica, Germany). Sections were double-stained with uranyl acetate and lead citrate followed by examination with TEM.

***Cq*VCP amino acids sequence alignment and RNA interference assay**

Signal sequence and domain prediction of *Cq*VCP were performed using SMART (http://www.smart.embl-heidelberg.de/). To explore effect of *Cq*VCP activity on WSSV infection, gene silencing of *CqVCP* was performed in Hpt cell culture. The *CqVCP* dsRNA was synthesized using MegaScript kit (Ambion, Austin, TX, USA)

1118 according to manufacturer's instructions and purified with TriPure Isolation Reagent
1119 (Roche, USA). DsRNA mixed with Cellfectin II Reagent (Life Techologies, USA)
1120 was transfected into 96-well plate of Hpt cell cultures (0.5 ug/well), which was
1121 repeated at 24 h after the first transfection. Efficiency of gene silencing was
1122 determined by qRT-PCR with GFP dsRNA treated samples as the control treatment.
1123 PCR primers were shown in Table 1.

1124 **Immunoblotting analysis**

1125 Hpt cell cultures were lysed in cell culture plates with 1×SDS-PAGE loading buffer
1126 after removing culture medium. Proteins samples were separated on SDS-PAGE gels
1127 and transferred to PVDF membranes. After blocking with skim 5% milk in
1128 TBST-buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Tween 20) for 1 h at
1129 25 °C, membranes were incubated with different primary antibodies for 2 h at 25 °C
1130 or 4 °C overnight, and subsequently incubated with the secondary antibody, *i.e.*
1131 alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies, for 2 h at
1132 25 °C followed by detection with an alkaline phosphatase substrate (Roche, USA).
1133 The conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was relatively quantified by
1134 ratio analysis on Western blotting signal of *Cq*GABARAP-II to *Cq*β-actin (60) unless
1135 otherwise stated. For immunoblotting on WSSV components, viral envelope protein
1136 was detected with a monoclonal antibody against VP28, or viral nucleocapsid protein
1137 VP664 with a multiple-clonal antibody against VP664 accordingly.

1138 **Proteins pull-down assay**

1139 Proteins pull-down assay was performed to explore interaction between *Cq*VCP

and *Cq*GABARAP. Full-length ORF of *Cq*VCP was recombinantly expressed with an N-terminal 6×His-tag in procaryotic expression system with *E. coli BL21* followed by purification with Ni-NTA agarose resin. Recombinant N-terminal GST-tagged r*Cq*GABARAP was purified with glutathione Sepharose 4B resin. Ten microgram of recombinant His-*Cq*VCP or rGST-*Cq*GABARAP were co-incubated in PBS buffer (pH 7.4) with end-over-end mixing at 4°C for 2 h, and followed by immobilization on 30 μL of glutathione Sepharose 4B resin. Binding proteins were resolved by 12% SDS-PAGE analysis and subjected to Coomassie blue staining, in which 0.2 μg of single protein was applied for the SDS-PAGE analysis accordingly.

Fluorescent intensity analysis and statistical analysis

All the experiments were performed at least three times unless otherwise stated. Student's *t*-test or one-way ANOVA was used for comparisons between different groups or treatments; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Fluorescent intensity, colocalization efficiency and Region-of-Interest (ROI) were processed using ImageJ software.

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FIGURE LEGENDS

FIG 1. Acidic vesicles were recruited for the viral fusion and intracellular trafficking of WSSV

(A) Endocytosis and intracellular trafficking of WSSV in the Hpt cells. A representative image shows the process of WSSV infection, including membrane invagination of WSSV (a), formation of intracellular endocytic vesicles containing WSSV (b), and transport of endocytic vesicles containing WSSV virions towards the perinuclear area (c, d). The WSSV virions are indicated by the white arrows. C: cytoplasm; N: nucleus.

(B) Real-time tracking of the intracellular trafficking of WSSV. Single WSSV virion labeled by DiD fluorescent dye (DiD-WSSV, red) was found to gradually accumulate in the Hpt cells over time, as determined by laser confocal microscopy examination. The white arrows indicate the gradual accumulation of WSSV virions within the cytoplasm in the Hpt cells. Time, 2 hpi.

(C) Intracellular trafficking of WSSV virions via acidic vesicles. Most of the endocytic virions, as indicated by the white arrows, are shown to move towards the perinuclear area over time. The WSSV virion was labeled with DiD fluorescent dye (red). The intracellular acidic vesicles were stained by Lysotracker (green).Time, 2 hpi.

(D) Fusion between the WSSV envelope and the membrane of acidic vesicles. The WSSV envelope was fused with the acidic vesicle membrane (lower), in which the fusion and dispersion of WSSV envelope proteins (white arrows) with the acidic vesicles were determined by the uniform colocalization of DiD and DiO fluorescence from the WSSV envelopes with the same vesicle compartment in time series.

(E) The time course observation on the detachment of the WSSV envelope form nucleocapsid. Most of the viral envelope was found to be detached from nucleocapsid within 2 hpi in Hpt cells. The intracellular WSSV virions were dually immune-stained with anti-VP28 monoclonal antibody against the envelope protein VP28 (red), and anti-VP664 antibody against the nucleocapsid protein VP664 (green), respectively

(the left). At least 120 WSSV-containing cells were counted per group with Hpt cell cultures (the right).

FIG 2. Alkalization of endosome acidity detained endocytic WSSV in the cytoplasm resulting in reduced viral infection

(A) Endocytic WSSV virions were trapped in dysfunctional endosomes induced by the alkalization. (a) The WSSV and the multiple vesicle bodies (MVBs) are indicated by white arrows and yellow arrows, respectively, with TEM. C: cytoplasm; N nucleus. (b) The isolated WSSV virions (red) were colocalized with endosomes (green) as identified by immunocytochemistry. The white arrows indicate colocalization between WSSV virion and endosome in the merged panel.

(c) The relative percent of colocalized WSSV with endosome was significantly higher in alkalized cells than it was in control cells by histogram analysis of the colocalization spots between VP28 and RabGEF1.

(B) Acidic endosomes were accumulated by alkalization in Hpt cells.

(a) Acidic endosomes, as indicated by white arrows, were dual-labelled with WGA and LysoTracker staining.

(b) Decreased fluorescence (Lysotracker staining) and (c) increased relative size of acidic endosomes (colocalization between WGA and Lysotracker staining) were induced by alkalization with chloroquine or NH_4Cl, respectively, for 1 h in the Hpt cells followed by examination with confocal microscopy, which were statistically analyzed and present by histogram analysis.

(C) Endocytic WSSV virions were detained in the alkalized endosomes as determined by real-time observation in live cells.

(a) Fusion and accumulation of endocytic WSSV virions with endosomes. In control cells, DiD-WSSV virions (red) were fused with acidic endosomes labelled by Lysotracker (green), and endosomes containing DiD-WSSV were gathered and accumulated as indicated by the yellow lines over time.

(b) Most of endocytic WSSV virions were isolated and detained in the alkalized endosomes. The Hpt cells were pretreated by chloroquine followed by infection with

WSSV labeled with DiD (red). The acidic endosomes were labelled by Lysotracker (green). White arrows indicate accumulated WSSV virions without fusion in the significantly enlarged endosomes caused by alkalization.

(c) Endocytic WSSV virions were accumulated and detained in enlarged endosomal vesicles induced by alkalization. DiD-WSSV and DiO-WSSV were accumulated in alkalized endosomes, in which the fusion between WSSV and endosomes was clearly reduced. The Hpt cells were pretreated by chloroquine followed by simultaneous infection with WSSV labeled with DiO (green) and WSSV labeled with DiD (red). Accumulated WSSV virions in endosomal vesicles are indicated by dashed circles over time.

(D) WSSV fusion was strongly inhibited by alkalizing acidic endosomes. Extensive colocalization fluorescence of WSSV envelope and nucleocapsid, with yellow signal as indicated by yellow arrows (left), was present in the Hpt cells treated by alkalizer chloroquine (CQ) or NH_4Cl. The relative percent of colocalized viral envelope with nucleocapsid was significantly higher in alkalized cells than it was in control cells by histogram analysis of the colocalization spots between VP28 and VP664 (right).

(E) Both degradation and replication of WSSV were reduced by alkalizing acidic endosomes. (a) Degradation of WSSV components was reduced by alkalizing acidic endosomes in Hpt cells. The degradation of WSSV components, as indicated by intracellular presence of viral envelope VP28 protein, was determined by immunoblotting against VP28 4 hpi in Hpt cells. (b) WSSV replication was significantly inhibited by alkalizing acidic endosomes in Hpt cells. (c) WSSV copy number was significantly reduced by alkalizing acidic endosomes in Hpt cells.

(F) Both fusion and replication of WSSV were not inhibited by blocking endosomal maturation. (a) Endosome maturation did not affect WSSV fusion. No significant inhibition of WSSV fusion was found in the YM-201636 treated cells in contrast to the positive control cells exposed to bafilomycin-A1 (Baf-A1). Hpt cells were pretreated with YM-201636 to block endosome maturation, or with bafilomycin-A1 to inhibit V-ATPase activity mediated acidity within endosomes, respectively, followed by WSSV infection. WSSV fusion was determined by immunofluorescence

1443 assay against the colocalization between viral envelope protein VP28 and
1444 nucleocapsid protein VP664. (b) Blocking endosome maturation did not inhibit
1445 WSSV replication. Blocking endosome maturation with YM-201636 did not result in
1446 clear inhibition of WSSV replication in Hpt cells (upper). In contrast, WSSV
1447 replication was significantly inhibited by bafilomycin-A1 exposure (lower).
1448 The TEM, confocal microscopy analysis and real-time imaging was taken from 3 hpi
1449 unless otherwise stated. WSSV replication and copy number were evaluated by
1450 relative gene expression of *VP28* transcript using qRT-PCR and by examination of
1451 VP28 DNA using PCR, respectively, 24 hpi. N.s, no significant difference; ** $p<0.01$;
1452 *** $p<0.001$.
1453
1454 **FIG 3.** WSSV replication via endosomal trafficking was disrupted by blocking
1455 *Cq*VCP ATPase activity
1456 (A) Both *CqVCP* gene and protein expression were responsive to WSSV infection *in*
1457 *vivo*. The transcript expression (a) and protein (b) level of *CqVCP* were profoundly
1458 induced in crayfish Hpt tissue *in vivo* after WSSV infection 1, 6 and 12 hpi .
1459 (B) WSSV infection was significantly inhibited by disrupting *Cq*VCP ATPase activity.
1460 (a) Both replication and degradation of WSSV were significantly decreased by
1461 *CqVCP* gene silencing. *CqVCP* gene silencing resulted in significant inhibition of
1462 *CqVCP* gene expression, which was accompanied with profoundly reduced viral
1463 replication (left). *Cq*VCP protein level was reduced by gene silencing of *CqVCP*
1464 followed by substantially decreased VP28 degradation (right).
1465 (b) Both replication and degradation of WSSV were significantly inhibited by
1466 blocking *Cq*VCP ATPase activity with DBeQ exposure.
1467 (C) Dynamic analysis of accumulated dysfunctional endosomes enclosing WSSV
1468 after disrupting *Cq*VCP ATPase activity. The observation of intracellular trafficking
1469 of WSSV over time was taken from 3 hpi in the Hpt cells. White arrows indicate
1470 colocalization of DiD-WSSV within acidic endosomes moving towards cell
1471 perinuclear area in control cells (upper). DiD-WSSV was colocalized within
1472 dysfunctional endosomes caused by disrupting *Cq*VCP ATPase activity with DBeQ

exposure, which further accumulated to form enlarged vesicles as indicated by white arrows (lower). Acidic endosomes were dual-labelled with WGA and LysoTracker staining.

(D) WSSV fusion was severely blocked in dysfunctional endosomes caused by disrupting *Cq*VCP ATPase activity. (a) *Cq*VCP accumulated and colocalized with WSSV aggregates in dysfunctional endosomes. *Cq*VCP was found to be colocalized with both RabGEF1 (upper) and WSSV by immunostaining against VP28 (lower) as indicated by yellow arrows in the Hpt cells after disrupting *Cq*VCP ATPase activity with DBeQ exposure. In contrast, no significant accumulated colocalization of *Cq*VCP and RabGEF1, or *Cq*VCP and WSSV was present in the DMSO treated control Hpt cells. (b) WSSV fusion was significantly inhibited by blocking *Cq*VCP ATPase activity. WSSV was colocalized with endosomes as immunostained by anti-RabGEF1 antibody (upper). Extensive colocalization of WSSV envelope protein VP28 and nucleocapsid protein (as indicated by immunostaining against VP664) was present in the Hpt cells lacking of *Cq*VCP ATPase activity caused by DBeQ exposure. No significant colocalization of VP28 and RabGEF1, or VP28 and VP664 was present in the DMSO treated control cells due to the on time degradation of the detached viral envelope. The colocalization was indicated by yellow arrows accordingly. Fluorescence intensity colocalization of VP28 and RabGEF1, or VP28 and VP664 was analyzed and shown in histogram analysis, respectively (right). (c) Aggregation of WSSV was strongly increased in dysfunctional endosomes caused by blocking *Cq*VCP ATPase activity. The ratio of WSSV aggregates (≥ 3 or < 3 virions per endosome) was relatively quantified with 80 cells containing WSSV at least in each group. Aggregated WSSV virions are indicated by white arrows, and endosomes containing WSSV virions are indicated by yellow arrows. These assays were performed 4 hpi.

(E) Both degradation and replication of WSSV were strongly inhibited by trapping of virions within dysfunctional endosomes caused by blocking *Cq*VCP ATPase activity. (a) Degradation of WSSV was strongly inhibited by blocking of *Cq*VCP ATPase activity. (b) Replication of WSSV was strongly inhibited by blocking of *Cq*VCP

ATPase activity. Hpt cells were pre-exposed to cycloheximide, to block proteins translation activity needing for viral replication, followed by WSSV infection.

(F) Propagation of progeny WSSV virions was abolished by blocking *Cq*VCP ATPase activity. In the upper, progeny WSSV virions, indicated by yellow arrows, were mostly present in nucleic area in the control Hpt cells 18 hpi, but fewer presence was found in the Hpt cells after blocking *Cq*VCP ATPase activity against DBeQ exposure. Segregated endocytic WSSV virions or remained viral components such as VP28 and VP664, indicated by white arrows, were mainly present in cytoplasm of the Hpt cells exposed to DBeQ (upper). In the lower, progeny virions, as indicated by yellow arrows, were found in the control cells but rarely in the Hpt cells exposed to DBeQ by TEM analysis 18 hpi (lower). The remained viral components were found to be present in the endocytic vesicles in cytoplasm, as indicated by white arrows, in DBeQ exposed cells.

WSSV replication was determined by quantification of the relative gene expression of *VP28* with qRT-PCR. The degradation of WSSV was determined with immunoblotting against presence of envelope protein VP28 with anti-VP28 monoclonal antibody. Intracellular localization of WSSV in Hpt cells was performed by TEM, in which *Cq*VCP ATPase activity was blocked by DBeQ exposure. N: nucleus; C: cytoplasm; N.s, no significant difference; * $p<0.05$; ** $p<0.01$, *** $p<0.001$.

FIG 4. Autophagy was induced by dysfunctional endosomes containing the aggregated WSSV virions caused by disrupting *Cq*VCP ATPase activity

(A) Autophagic activity was increased by *CqVCP* gene silencing in Hpt cells. Autophagic activity, as indicated by the conversion of *Cq*GABARAP-I (*Cq*GB-I) to *Cq*GABARAP-II (*Cq*GB-II), was profoundly increased by gene silencing of *CqVCP*.

(B) Autophagic activity was induced by blocking *Cq*VCP ATPase activity in Hpt cells.

(a) *Cq*GABARAP puncta formation was increased by blocking *Cq*VCP ATPase activity with DBeQ exposue in a dose-dependent manner in Hpt cells. (b) Intensity of

fluorescent *Cq*GABARAP puncta in (a) was relatively calculated and shown by histogram analysis. (c) The conversion of *Cq*GABARP-I to *Cq*GABARP-II was significantly increased by blocking *Cq*VCP ATPase activity with DBeQ exposue in a dose-dependent manner in Hpt cells. (d) Aggregated WSSV virions were surrounded by substantially accumulated *Cq*GABARAP after blocking *Cq*VCP ATPase activity. Aggregated WSSV virions, as indicated by fluorescent viral VP28 protein, were surrounded by increased accumulation of *Cq*GABARAP puncta in the Hpt cells pre-exposed to DBeQ in a dose dependent manner.

(C) Dysfunctional endosomes containing WSSV aggregates fused with autophagosomes due to a lack of *Cq*VCP ATPase activity. (a) Dysfunctional endosomes caused by blocking *Cq*VCP ATPase activity fused with autophagosomes. As indicated by yellow arrows, blocking of *Cq*VCP ATPase activity resulted in profound *Cq*VCP accumulation within autophagosomes (upper) and fusion of accumulated endosomes with autophagosomes (lower). (b) Dysfunctional endosomes containing aggregated WSSV virions fused with autophagosomes. Yellow arrows indicate colocalization of *Cq*VCP and *Cq*GABARAP, *Cq*GABARAP and WGA, WGA and VP28, or *Cq*GABARAP and VP28, respectively. Autophagosome localization was indicated by accumulation of *Cq*GABARAP puncta (green). Endosomes localization was indicated by WGA staining (red in a; green in b).

(D) Bioinformatics analysis of *Cq*VCP amino acid sequences and its binding to *Cq*GABARAP. (a) Conserved domains and putative LIR motifs in *Cq*VCP. LIRs motifs, as indicated by red boxes, locates in CDC48-2 domain of *Cq*VCP similar to human VCP LIRs (gene ID 7415, Genbank No. NM_007126.5). (b) *Cq*VCP bound to *Cq*GABARAP. Binding proteins were determined by Coomassie brilliant blue staining.

(E) Initiation of autophagy induced by blocking *Cq*VCP ATPase activity was dependent on V-ATPase activity of endosomes. (a) Initiation of autophagy induced by blocking *Cq*VCP ATPase activity was disrupted by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873. Dysfunction of *Cq*VCP ATPase activity by DBeQ significantly induced the autophagic activity, in which the autophagy induced by

DBeQ could be blocked by pre-binding of NMS873 to *Cq*VCP. Hpt cells were exposed to NMS873 for 2 hours followed by exposure against DBeQ for 4 hours. (b) Activation of autophagy associated with dysfunctional *Cq*VCP was dependent on endosomal V-ATPase activity. Endosomal V-ATPase activity was inhibited by pretreatment with bafilomycin-A1 in Hpt cells, which blocked the lipidation of *Cq*GABARAP caused by dysfunctional *Cq*VCP ATPase activity with DBeQ exposure. V-ATPase activity of intracellular endosomes was inhibited by bafilomycin-A1 for 2 hours in the Hpt cells, which was followed by exposure to DBeQ for 4 hours. (c) *Cq*VCP-associated autophagy was dependent on V-ATPase activity. Formation of *Cq*GABARAP puncta induced by *Cq*VCP-DBeQ complex was significantly reduced by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873 or by inhibition on V-ATPase with bafilomycin-A1.

The conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was determined by immunoblotting with anti-*Cq*GABARAP antibody. *Cq*VCP ATPase activity was blocked by DBeQ exposure in Hpt cells. The confocal microscopy analysis with WSSV infection was taken from 4 hpi unless otherwise stated. N.s, no significant difference; * $p<0.05$; ** $p<0.01$.

FIG 5. Increased autophagic activity substantially reduced WSSV infection

(A) Autophagy was increased by WSSV infection. Both protein expression of *Cq*GABARAP and the conversion of *Cq*GABARAP-I to *Cq*GABARAP-II were induced by WSSV infection (left). The relative quantitation of the conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was determined by histogram analysis (right).

(B) Autophagy was induced by AKTi 1/2 exposure in Hpt cells. (a) Formation of *Cq*GABARAP puncta was increased against AKTi 1/2 exposure in a dose dependent manner. (b) Statistic analysis of fluorescent *Cq*GABARAP puncta formation. Increased fluorescence of *Cq*GABARAP puncta in (a) was relatively quantified and present by histogram analysis. (c) Significant conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was induced by AKTi 1/2 in a dose dependent manner.

(C) Real-time observation on increased accumulation and fusion of the

WSSV-containing endosomes caused by induced autophagic activity. Fusion of WSSV-containing endosomes is indicated by white arrows in the control cells (upper). Accumulation of WSSV-containing endosomes, as indicated by yellow arrows, was clearly increased by induced autophagic activity against AKTi 1/2 exposure in the Hpt cells (lower). Hpt cells were infected by WSSV labeled with DiD, and endosomes were labelled by WGA staining. Time, 3 hpi.

(D) WSSV envelope fusion was significantly inhibited by the increased autophagic activity. (a) Accumulation and fusion of WSSV-containing endosomes with autophagosomes was increased by the induced autophagic activity. More endosomes containing WSSV virions were accumulated and fused with each other to form the enlarged vesicles as indicated by yellow arrows (upper), which were surrounded by autophagosomes as indicated by yellow arrows (lower) in Hpt cells with the increased autophagic activity by AKTi 1/2 exposure. Colocalization of endosome with WSSV (upper) or autophagosome and endosomes (lower), as indicated by yellow arrows, was increased by induced autophagic activity. (b) Release of WSSV nucleocapsid was significantly inhibited by the increased autophagic activity. Colocalization of autophagosome with WSSV (upper) or VP28 with VP664 (lower), as indicated by yellow arrows, was significantly increased in Hpt cells with higher autophagic activity induced by AKTi 1/2 exposure (left), in which the relative colocalization efficiency was determined by histogram analysis (right). In (a) and (b), WSSV was localized with anti-VP28 antibody against viral envelope protein or with anti-VP664 antibody against nucleocapsid protein. Endosomes were immunostained by anti-RabGEF1 antibody or labeled with WGA fluorescent dye, respectively. Autophagosomes were localized with anti-*Cq*GABARAP antibody. (c) Accumulation of endocytic WSSV virions in autophagosomes was increased by the induced autophagic activity. Autophagosomes with double-layer membranes are indicated by yellow arrows, in which the enclosed WSSV virions are indicated by white arrows (left). Number of WSSV-containing autophagosomes, with less or more than 3 virions per autophagosome, is calculated in AKTi 1/2 exposed cells and control cells, respectively, shown by histogram analysis (right). Hpt cells were pre-exposed with

AKTi 1/2 followed by WSSV infection. Cell samples were analyzed by TEM, in which the number of WSSV-containing autophagosomes was defined as <3 and ≥3 virions per autophagosome, respectively.

(E) Reduced WSSV replication due to the increased viral degradation by induced autophagic activity. (a) Hpt cells were pre-exposed to cycloheximide (CHX) to block protein translation activity, or to AKTi 1/2 to increase autophagic activity as indicated, which were subsequently infected with WSSV. Presence of WSSV components was determined by immunoblotting against viral envelope protein VP28. (b) WSSV replication was evaluated by relative gene expression of *VP28* in Hpt cells using qRT-PCR.

(F) Both acidity and acidic enzyme activity were significantly increased in Hpt cells by the induced autophagic activity. In upper panel, accumulation of autophagosomes and acidity of intracellular acidic vesicles, including autophagosome, were strongly increased by the induced autophagic activity with AKTi 1/2 exposure in Hpt cells (left). Acidic vesicles were stained with LysoTracker. White arrows indicate accumulated acidic vesicles, which are likely to increase the efficiency of autophagic degradation. Histograms show substantially increased accumulation of autophagosomes induced by the increased autophagy activity (right). In lower panel, fluorescent signal of cathepsin-magic dye, indicating the acidity-dependent enzymes activity such as cathepsin L activity in intracellular acidic vesicles, was profoundly increased in Hpt cells with the induced autophagic activity by AKTi 1/2 exposure. White arrows indicate accumulated acidic vesicles with higher cathepsin L enzyme activity, which was likely to induce the efficiency of autophagic degradation (left). Histograms show the significantly higher fluorescent intensity of acidic vesicles induced by the increased autophagy activity (right). The confocal microscopy analysis with WSSV infection was taken from 4 hpi unless otherwise stated. N.s, no significant difference; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

FIG 6. WSSV escaped from autophagic elimination via direction to endosomal delivery system substantially facilitated by *Cq*VCP

(A) Most of endocytic WSSV virions escaped from autophagy degradation via direction to endosomes facilitated by *Cq*VCP. (a) Dysfunctional endosome, via blocking *Cq*VCP ATPase activity, resulted in more WSSV aggregation than that by increasing autophagic activity. Aggregated WSSV virions were localized by immunostaining against viral envelope protein VP28 as indicated by white arrows. *Cq*VCP ATPase activity was blocked by DBeQ exposure and increased autophagic activity was induced by AKT i1/2 exposure in Hpt cells. (b) Relative quantification of the integrated optical density of fluorescent VP28 signal in upper of (a). (c) Relative quantification of colocalization of VP28 and VP664. Histogram analysis shows integrated optical density of the colocalized fluorescent signals of VP28 and VP664, as indicated with yellow arrows, in Hpt cells in lower of (a). (d) Dysfunctional endosomes, via blocking *Cq*VCP ATPase activity, resulted in more inhibition on WSSV replication than that by increasing autophagic activity. Relative gene expression of WSSV was determined by presence of *VP28* transcript 24 hpi in Hpt cells using qRT-PCR.

(B) Homotypic fusion of WSSV-containing endocytic vesicles and endosomes were significantly inhibited by restricting D2 in *Cq*VCP in an ADP-bound state.

(a) WSSV envelope fusion was profoundly inhibited by blocking homotypic fusion of WSSV-containing endocytic vesicles and endosomes. In upper panel, inhibition of viral fusion was indirectly indicated by fluorescent presence of WSSV envelope protein VP28 as indicated by white arrows. In contrast to the control Hpt cells after the viral fusion, much more fluorescent viral envelope protein such as VP28 was present in the cells lacking of *Cq*VCP activity caused by NMS873 exposure (left). Histogram analysis shows fluorescence intensity of intracellular viral envelope VP28 protein (right). In lower panel, homotypic fusion was abolished by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873 exposure in Hpt cells followed by alkalization with chloroquine. In contrast to the control Hpt cells after the viral fusion, rarely accumulated endosome containing WSSV was present in the cells lacking of *Cq*VCP activity caused by pre-exposure to NMS873. Strongly accumulated colocalization of endosome and WSSV is indicated by yellow arrows in the control

cells without NMS873 exposure (left). Histogram analysis shows fluorescence intensity of colocalization of RabGEF1 and VP28 (right).

(b) Increased autophagic activity induced by WSSV was in a viral dose dependent manner in Hpt cells after blocking homotypic fusion of WSSV-containing vesicles and endosomes. The conversion of *Cq*GABARAP-I to *Cq*GABARAP-II was in a WSSV dose dependent manner in Hpt cells after restricting D2 in *Cq*VCP in an ADP-bound state with NMS873 exposure. Hpt cells were pre-exposed to NMS873 followed by WSSV infection with different MOIs as indicated.

(C) Direction of WSSV to autophagosomes was inhibited by blocking homotypic fusion of endocytic vesicles and endosomes.

(a) Restricting D2 in *Cq*VCP in an ADP-bound state exposed to NMS873 did not inhibit conversion of *Cq*GABARAP-I to *Cq*GABARAP-II induced by exposure to AKTi 1/2. (b) Both *Cq*GABARAP puncta formation and acidic vesicles were substantially reduced by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873 exposure. Reduced *Cq*GABARAP puncta formation and acidic vesicles indicated inhibited fusion of endosomes and autophagosomes by blocking *Cq*VCP activity with NMS873 exposure. *Cq*GABARAP puncta immunostained by anti-GABARAP antibody are indicated by white arrows in upper. Acidic vesicles stained by LysoTracker are indicated with green fluorescence in lower.

(c) Direction of WSSV to autophagosomes was inhibited by blocking homotypic fusion of endocytic vesicles and endosomes. Strongly accumulated WSSV virions in autophagosomes are indicated by yellow arrows in the control cells solely exposed to AKTi 1/2, but much less accumulation with small size was present in the cells pre-exposed to NMS873 followed by AKTi 1/2 exposure (left). Histogram analysis shows significant inhibition of direction of WSSV to autophagosomes as indicated by colocalized fluorescence intensity of VP28 and *Cq*GABARAP (right). Hpt cells were pre-exposed to NMS873 for 2 hours followed by AKTi 1/2 exposure for 4 hours and infected by WSSV.

(D) WSSV replication was profoundly inhibited by disruption of *Cq*VCP mediated homotypic fusion of endocytic vesicles and endosomes. Hpt cells was pre-exposed to

NMS873 followed by WSSV infection. Both WSSV replication and translation were substantially inhibited by restricting D2 in *Cq*VCP in an ADP-bound state with NMS873 exposure. Viral gene replication and protein synthesis were determined by qRT-PCR and Western blotting accordingly.

(E) Propagation of progeny WSSV virions was strongly abolished by disrupting homotypic fusion of endocytic vesicles and endosomes mediated by *Cq*VCP.

In upper panel, progeny virions were mostly present in the nucleic area in the control Hpt cells 18 hpi, but was rarely found in the Hpt cells after blocking homotypic fusion by restricting D2 in *Cq*VCP in an ADP-bound state by NMS873 exposure. Progeny virions are indicated by yellow arrows with immunostaining in the control cells. Segregated endocytic WSSV virions or remaining viral components are indicated by white arrows in cytoplasm of the Hpt cells exposed to NMS873, in which the remaining viral components such as VP28 were present only in cytoplasmic area as indicated by white arrows (left). Histogram analysis shows relative percent of Hpt cells containing progeny virions (right). Hpt cells containing progeny WSSV virions were relatively quantified by counting immunostaining signals of colocalization of VP28 and VP664 in cell nucleus.

In lower panel of TEM analysis, progeny virions were found only in the control cells but not in NMS873 exposed Hpt cells 18 hpi. Progeny virions are indicated by yellow arrows in control cells. Remained viral components were found to be present in endocytic vesicles in cytoplasm. Homotypic fusion of endocytic vesicles and endosomes was disrupted by pre-exposure to NMS873 for 2 hours in Hpt cells followed by WSSV infection. N: nucleus; C: cytoplasm.

The confocal microscopy analysis with WSSV infection was taken from 4 hpi unless otherwise stated. N.s, no significant difference; ** $p<0.01$; *** $p<0.001$.

FIG 7. Schematic model of cytoplasmic trafficking of WSSV for infection.

Table 1
Primer sequences used in this study

Primer	Sequence (5'-3')
Gene cloning	
CqVCP-RACE-F	CTGCATCTTCCACGATGAGGCGGTTTGG
CqVCP-RACE-R	CGCAAATATGAGATGTTCTCCCAGACGC
CqVCP ORF-F	ATGGCCGAACAGGAAGACTTAGC
CqVCP ORF-R	CAAACCACTCATGAATGGTACACTAAT
RNA interferance	
ds*CqVCP*-F	TAATACGACTCACTATAGGGGCCCTTCAACAAATCAGAGA
ds*CqVCP*-R	TAATACGACTCACTATAGGGCAACTCGTCAAAGAACAAGACACAC
ds*GFP*-F	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT
ds*GFP*-R	TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC
16s-F	AATGGTTGGACGAGAAGGAA
16s -R	CCAACTAAACACCCTGCTGATA
CqVCP-F	GCGAATCCACACTAAGAACAT
CqVCP-R	AGCATCAATCTGGTCATCATC
VP28-F	AAACCTCCGCATTCCTGT
VP28-R	GTGCCAACTTCATCCTCATC