White spot syndrome virus entry is dependent on multiple endocytic routes and strongly facilitated by Cq-GABARAP in a CME-dependent manner

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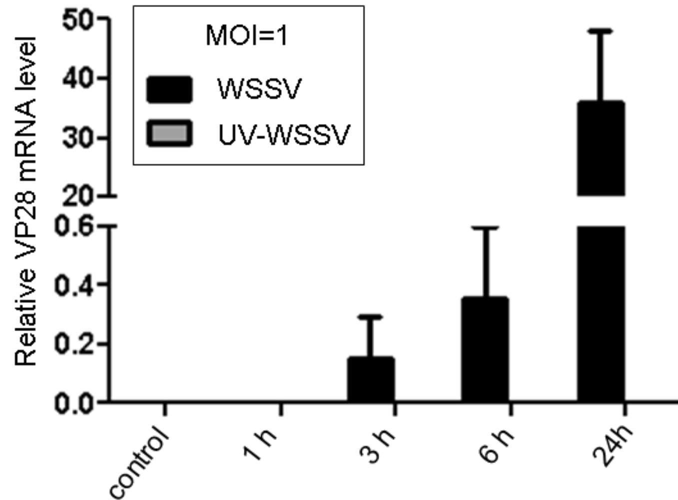
Running title: WSSV entry mechanism

Supplementary Figures 1 to 3, Table 1, and Methods



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1085 **Supplementary Figure 1. Examination of the expression of the WSSV envelope**
1086 **protein VP28 during viral infection in Hpt cells by qRT-PCR.** The Hpt cells were
1087 incubated with indicated virus inoculation, namely WSSV or UV-inactivated WSSV
1088 (UV-WSSV), or uninfected as control. After indicated time points (1, 3, 6 and 24 hpi),
1089 the cells were harvested for qRT-PCR, showing that no VP28 transcription was
1090 detected in Hpt cells uninfected or infected with UV-WSSV, while increased amount
1091 of VP28 translation since 3 hpi in the cells infected with WSSV.

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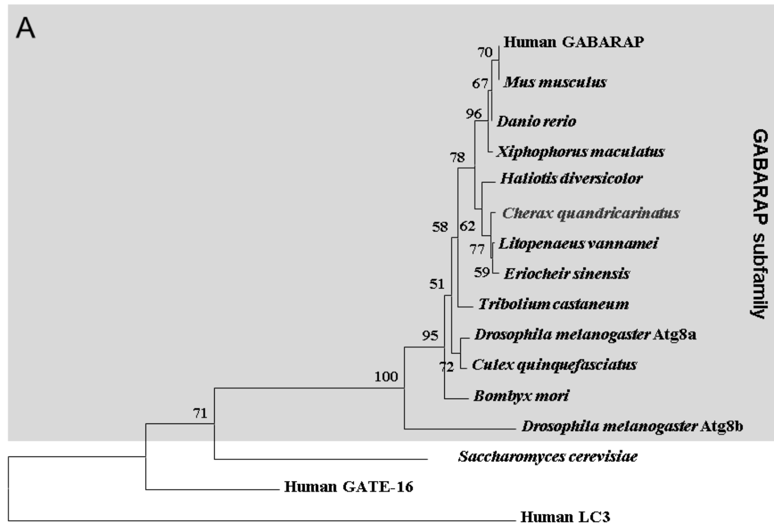

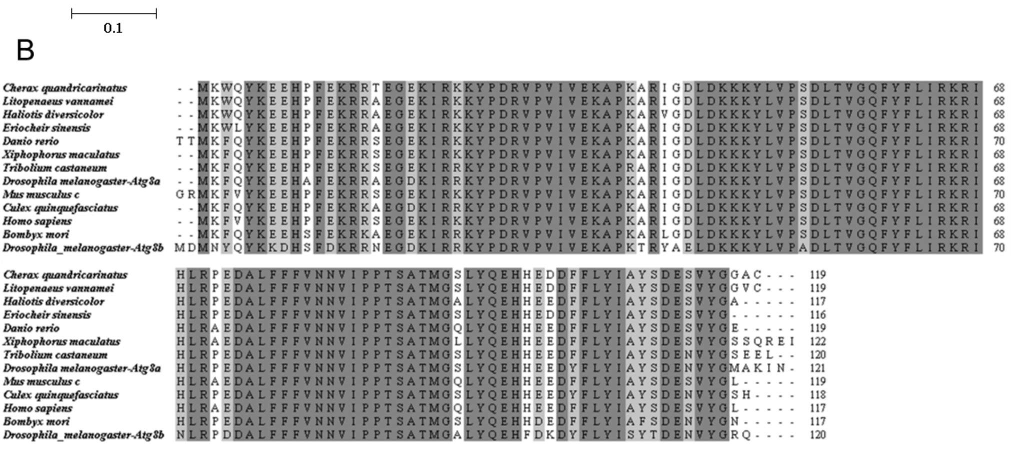

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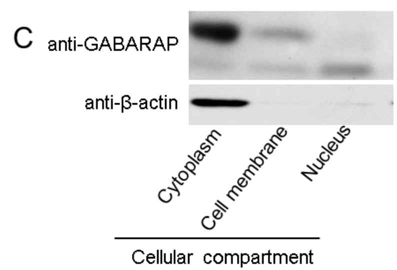

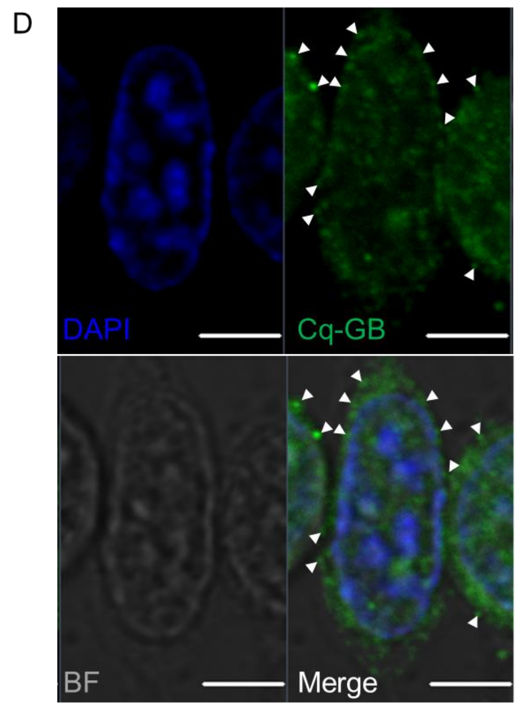

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1101 **Supplementary Figure 2. Characterization of Cq-GABARAP.** A: Phylogenetic

1102 tree of Atg8 family proteins. B: Multiple sequence alignment of GABARAP from
1103 different species. The identical amino acid residues are dark gray, and similar amino
1104 acids are shown with a light gray background (*Litopenaeus vannamei*, AFV99179.1;
1105 *Haliotis diversicolor*, ADI56518.1; *Eriocheir sinensis*, AEE60804.1; *Danio rerio*,
1106 XP_003199067.2; *Xiphophorus maculatus*, XP_005804839.1; *Tribolium castaneum*,
1107 XP_973073.1; *Culex quinquefasciatus*, XP_001844428.1; *Drosophila*
1108 *melanogaster*-Atg8a, NP_727447.1; *D. melanogaster*-Atg8b, AAF55459.1; *Bombyx*
1109 *mori*, NP_001040244.1; *Mus musculus*, EDL12513.1; *Homo sapiens*, NP_009209.1;
1110 Human GATE-16, P60520; Human LC3, Q9H492; Human GABARAP,
1111 NP_009209.1). C: The distribution of Cq-GABARAP protein in different cellular
1112 compartments examined by immunoblotting. β-actin was used as a marker protein for
1113 the cytoplasmic compartment. D: Distribution of Cq-GABARAP in Hpt cells
1114 determined by IFA. Hpt cells were harvested for IFA analysis with anti-GABARAP
1115 antibody. The nucleus was stained with DAPI and cell images were taken under
1116 confocal microscopy. White arrowheads indicate some of the events that
1117 Cq-GABARAP located on cell membranes. Bars indicate 10 μm.
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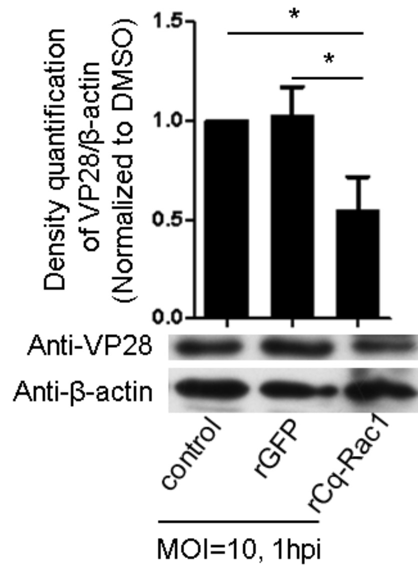

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1120 **Supplementary Figure 3. Suppression of WSSV entry by recombinant Cq-Rac1**
1121 **(rCq-Rac1).** Hpt cell cultures were transfected with rCq-Rac1 (1 μg), rGFP or no
1122 protein as a control for 40 min using PULSin and then subjected to viral infection
1123 before viral entry examination by immunoblotting (lower panel). The band intensities
1124 of three independent experiments were analyzed using the QuantityOne program
1125 (upper panel). The data are presented as the mean ± SEM from at least three
1126 independent experiments and were analyzed by Student's *t* test (*$P<0.05$).

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1134 **Supplementary Table 1. Primers used in the present study**

Primers	Sequence information (5'–3')
Gene clone	
5’CLC 612-	CTTGATGGGAGGATTCTGCTTCA
3’CLC 210+	TTGAGATGCTGGGTGGAGATGA
Degenerate AP50+	TAYAAYCAYAARGGIGARGT
Degenerate AP50-	CCRAAYTTRCAYTCIGGCAT
5 AP50 357-	TGTGTCTGTGTTCTGAGGATACCCA
3 AP50 332+	TTGGGTATCCTCAGAACACAGACAC
5’GB 199-	ATGGTAGCGGAGGTTGGAGGAATGA
3’GB +	GTAGCGGAGGTTGGAGGGATGAC
Rac1+	CGAGGATGGAGGATTGTTGAG
Rac1-	TCGCTTGACTGGGAATCTGAG
5DYN 338-	TTCTGACCCGTAATGCGATCTGTG
3DYN 2085+	GCTCCTTGCCCACCTGTATGCC
qRT-PCR	
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC
VP28-F	AAACCTCCGCATTCCTGT
VP28-R	GTGCCAACTTCATCCTCATC
16s-F	AATGCTTCTTGCACCACCAAC
16s-R	AGGTCTTGCTCAGCTGGATACC
Dete-CLC+	TGCTGAGGAGGTAAGCAAGATAG
Dete-CLC-	GCATTTAAGCCTTGATGGGAG
Dete-AP50 499+	AAGACAGCAAGCAAGGAGGAG
Dete-AP50 889-	CTGGAGGAACAAATGAAATAGAGTG
Dete-GB155+	CGTGCCGGTAATTGTAGAGAAGG
Dete-GB594-	AAACTGCACCAAGACACCCTG
dete Dyn 1853+	CATTCCTACGTGCTGGTGTTT
dete Dyn 2160-	TCGTTTACGTGCCTCCTCTGG
RNAi	
Ri-CLC+	TAATACGACTCACTATAGGGCGACCATCACACACCATCTACAC
Ri-CLC-	TAATACGACTCACTATAGGGTCTGTGCCTGGTTCCATCTTC
RiAP50 226+	TAATACGACTCACTATAGGGACGAACATTGCACGCACCA
RiAP50 669-	TAATACGACTCACTATAGGGCGCCACATGGGCAGACAA
Ri-GB 188+	TAATACGACTCACTATAGGGTCGCATTGGTGACTTGGATAAG
Ri-GB 639-	TAATACGACTCACTATAGGGGCAATTCTTTAGCACCTCAGGG
Ri Dyn 2055+	TAATACGACTCACTATAGGGCACGTCTATGGACCCTCAACT
Ri Dyn 2592-	TAATACGACTCACTATAGGGAAGAATCCCTGAATTACCCTGT
Recombinant expression	
eGB 1+	CGGGATCCATGAAGTGGCAGTACAAGG
eGB 396-	AAGCGGCCGCTCAGCACGCCCCTCCATA
eRac1 1+	GGAATTCCATATGATGTCATCTGGCCGTCCCAT
eRac1 588	CCGGAATTCTTAGAGGACTGAGCACTTGCGC

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1136 ## Supplementary Methods

1137 **Hpt cell cultures.** The hematopoietic tissue from the dorsal side of the crayfish
1138 stomach was dissected, briefly washed with CPBS (crayfish phosphate buffer saline:
1139 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 mM $CaCl_2$, 10 mM $MnCl_2$ and
1140 27 mM KCl, pH 6.8) and incubated in 500 μl of collagenase (0.1% collagenase type I
1141 and IV in CPBS) for 45 min at room temperature to dissociate the Hpt cells. The
1142 separated cells were washed twice with CPBS and then centrifuged for 3 min at 1,500
1143 *g* and room temperature. The cell pellet was resuspended in modified L15 medium
1144 (Sigma-Aldrich) containing 5 μM 2-mercaptoethanol (Sigma-Aldrich), 1 μM
1145 phenylthiourea (Sigma-Aldrich), 60 μg/ml penicillin (Gibco), 50 μg/ml streptomycin
1146 (Gibco), 50 μg/ml gentamicin (Gibco) and 2 mM L-glutamine (Thermo Fisher) and
1147 then subsequently seeded at a density of 2×10^5 cells per 0.5 ml in 24-well plates. The
1148 prepared Hpt cells were allowed to attach for 30 min at room temperature, and 2 μl of
1149 a plasma preparation containing crude astakine was then added to each well as
1150 described by Söderhäll et al.[1]. One-third of the medium was replaced with fresh
1151 medium every second day.

1152 **Total RNA isolation and qRT-PCR:** The Hpt cells were harvested for total RNA
1153 isolation using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) at 6 hpi.
1154 After DNase I (Ambion) digestion, 1 μg of the purified total RNA was used for cDNA
1155 synthesis with the Prime Script™ RT Reagent kit (TaKaRa). qRT-PCR was performed,
1156 and the transcripts for IE1 (GenBank accession no. NC_003225.2) and VP28

1157 (GenBank accession no. AF227911.1) were employed to assess the replication of
1158 WSSV in Hpt cell cultures. The crayfish 16S ribosomal RNA gene (GenBank
1159 accession no. AF135975.1)[2], a mitochondrial ribosomal RNA gene[3,4], was utilized as
1160 an internal control.

1161 **Immunoblotting analysis:** The protein samples were separated by 13.5%
1162 SDS-PAGE and wet-transferred to a PVDF membrane. The membrane was blocked in
1163 5% non-fat milk in TBST for 2 h, briefly washed with TBST, incubated with a 1:3000
1164 dilution of the anti-VP28 primary antibody, and washed three times with TBST for 8
1165 min/wash. The membrane was then incubated with an HRP-conjugated
1166 goat-anti-mouse IgG antibody at a dilution of 1:10,000 in TBST for 1 h, washed five
1167 times with TBST (8 min/wash) and detected using the ECL reagent (Minipore). The
1168 immunoblotting signal of protein bands was quantified with QuantityOne software
1169 (Bio-Rad).

1170 **Gene cloning: Cq-GABARAP, Cq-CLC, Cq-AP50, Cq-dynamin and**
1171 **Cq-RAC1.** We previously isolated partial sequences of the Cq-GABARAP and
1172 Cq-CLC genes, which are transcriptionally up-regulated in Hpt cells following WSSV
1173 challenge[5]. Full-length cDNAs encoding the open reading frames of these genes were
1174 obtained by RACE-PCR. To clone other evolutionally conserved molecules involved
1175 in the CME process, such as Cq-AP50, Cq-dynamin and Cq-Rac1, degenerate PCR
1176 followed by RACE-PCR was performed to obtain the full-length cDNAs for these
1177 genes. All of the primers used for PCR are shown in Supplementary Table 1. The

1178 BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program was used to analyze the
1179 sequences, and Bioedit 7.0.9.0 and Mega 5.0 were used to conduct the multiple
1180 sequence alignments and phylogenetic analysis, respectively.

1181 **Preparation of the recombinant proteins.** The open reading frame of
1182 Cq-GABARAP or Cq-CLC was cloned into the pGEX-4T-2 bacterial expression
1183 vector (GE Healthcare). The open reading frame of Cq-Rac1 or GFP was cloned into
1184 the pET28a bacterial expression vector (GE Healthcare) using primers as listed in
1185 Supplementary Table 1. The correct orientation and sequence of the cloned genes
1186 were confirmed by sequencing. The resulting plasmids were then introduced into *E.*
1187 *coli* (BL21), and IPTG was used to induce the ectopic expression of the inserted genes.
1188 The recombinant GST-fusion proteins were purified using an ÄKTA system with a
1189 GSTrap FF column (GE Healthcare) followed by dialysis against phosphate buffered
1190 saline (PBS, pH 7.4) to remove the excess GSH. The concentration of the resulting
1191 proteins was then determined, and the proteins were mixed with an equal volume of
1192 glycerol and stored at -80 ℃ for later use.

1193 **Biotinylation of WSSV:** To isolate the putative viral binding proteins from the
1194 Hpt cells, the WSSV envelope proteins were biotinylated by incubating the freshly
1195 prepared viral envelope proteins with NHS-D-biotin (Sigma) at room temperature for
1196 4 h. Ammonium chloride (NH_4Cl) was then added to the reaction solution to stop the
1197 labeling reaction, and the mixture was then dialyzed three times with PBS buffer (12

1198 h/time) at 4 ℃ to remove the excess biotin. The obtained biotinylated viral envelope

1199 proteins were used for the pull-down assays.

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