PmAP2-β depletion enhanced activation of the Toll signaling pathway during yellow head virus infection in the black tiger shrimp *Penaeus monodon*

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Yellow head virus (YHV) is a pathogen which causes high mortality in penaeid shrimp. Previous studies suggested that YHV enters shrimp cells via clathrin-mediated endocytosis. This research investigated the roles of clathrin adaptor protein 2 subunit β (AP-2β) from *Penaeus monodon* during YHV infection. *Pm*AP2-β was continuously up-regulated more than twofold during 6–36 hpi. Suppression of *Pm*AP2-β significantly reduced YHV copy numbers and delayed shrimp mortality. Quantitative RT-PCR revealed that knockdown of *Pm*AP2-β significantly enhanced the expression level of *Pm*Spätzle, a signaling ligand in the Toll pathway, by 30-fold at 6 and 12 hpi. Moreover, the expression levels of gene components in the Imd and JAK/STAT signaling pathways under the suppression of *Pm*AP2-β during YHV infection were also investigated. Interestingly, anti-lipopolysaccharide factor isoform 3 (ALFPm3) was up-regulated by 40-fold in *Pm*AP2-β knockdown shrimp upon YHV infection. In addition, silencing of *Pm*AP2-β dramatically enhanced crustin*Pm*1 expression in YHV-infected shrimp. Knockdown of ALFPm3 and crustin*Pm*1 significantly reduced shrimp survival rate. Taken together, this work suggested that *Pm*AP2-β-deficiency promoted the Toll pathway signalings, resulting in elevated levels of ALFPm3 and crustin*Pm*1, the crucial antimicrobial peptides in defence against YHV.

**Abbreviations**

ALFs  Anti-lipopolysaccharide factors  
AMPs  Antimicrobial peptides  
Imd  Immune deficiency  
*Pm*  *Penaeus monodon*  
WSSV  White spot syndrome virus  
YHV  Yellow head virus

Yellow head virus (YHV) is a lethal positive-sense single-stranded RNA virus with a spike envelope. YHV widely infects penaeid shrimps, including *Euphausia superba*, *Litopenaeus setiferus*, *P. merguiensis*, *Metapenaeus ensis*, *L. vannamei*, *P. stylirostris*, *P. setiferus*, *P. aztecs* and *P. duorarum*¹⁻³. YHV entry via the clathrin-mediated endocytosis has been identified by endocytosis inhibition and by silencing of the clathrin coated assembly protein

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AP2-β transcript can be efficiently suppressed by Pm on signaling pathways and other immune-related genes during YHV infection. In previous research, we have presumably these Pm A virus12, Foot- and mouth disease virus13 and hepatitis C14, hijack the clathrin-mediated endocytosis to enter microsome (TEM). As shown in Fig. 2A–D, conjugated with Pm infected shrimp hemocytes. In addition, clusters of Pm was reported to have been governed by the Toll and the Imd pathways26. Crustin Deficiency (Imd) signaling pathways are one of the first lines of shrimp innate immunity. Previously, ALF Pm The expression of antimicrobial peptides was controlled by different signaling pathways. The Toll and Immune pathways.

Results

Effect of PmAP2-β silencing on the Toll, the Imd and the JAK/STAT signaling pathways during YHV infection. As shown in Fig. 3A,B, YHV infection significantly enhanced the transcription of PmSpätzle and myeloid differentiation factor 88 (MyD88) in the Toll pathway. PmSpätzle was increased by 12, 16, 7, 10, 10, 3-fold at 6, 12, 18, 24, 30 and 36 hpi, respectively, while PmMyD88 gradually increased and reached the highest level (sixfold) at 18 hpi. This suggested that the Toll signaling pathway responded to YHV infection. Notably, expression of PmDorsal in YHV-challenged shrimp remained at a similar level, compared with that in non-infected shrimp (Fig. 3C).

Next, the RNA interference experiment was carried out in order to investigate the influence of PmAP2-β on signaling pathways and other immune-related genes during YHV infection. In previous research, we have shown that PmAP2-β transcript can be efficiently suppressed by PmAP2-β dsRNA7. Interestingly, expression of PmSpätzle in PmAP2-β silenced shrimp challenged with YHV was highly up-regulated by 31- and 33-fold.
Figure 1. Expression of PmAP2-β and effect of PmAP2-β silencing on YHV infection. (A) The relative transcription levels of PmAP2-β during YHV infection. The mRNA expression levels of PmAP2-β were analyzed by Quantitative Real-time RT-PCR. The experiment was carried out in triplicate. (B) Expressions of PmAP2-β (green) and gp116 (red) in the hemocytes of unchallenged and YHV-challenged shrimp were observed by confocal laser scanning microscopy. Secondary antibodies conjugated with Alexa Fluor 488 (green) and Alexa Fluor 568 (red) were used to probe anti-AP2-β and gp116 antibodies, respectively, while nuclei were stained in blue. (C) Effect of PmAP2-β silencing on cumulative mortality caused by YHV. Shrimp were injected with either 150 mM NaCl or 10 µg GFP dsRNA per 1 g of shrimp or 10 µg PmAP2-β dsRNA per 1 g of shrimp at 24 h prior to YHV challenge. The cumulative mortality was recorded every 12 h after YHV injection. Each group contains 10 shrimps. The experiment was carried out in triplicate. (D) Determination of YHV copy number in PmAP2-β knockdown shrimp. Shrimp were divided into three groups and injected with either 150 mM NaCl, GFP dsRNA (10 µg/g shrimp), PmAP2-β dsRNA (10 µg/g shrimp). Shrimp hemocytes were collected at 6, 12, 18, 24, 30 and 36 h after YHV injection for YHV copy number determination. The data are shown as the mean ± standard deviation. An asterisk represents significant differences from control group (p < 0.05). The experiment was carried out in triplicate.
at 6 and 12 hpi, compared with non-infected shrimp (Fig. 3A). In addition, *PmAP2-β* silencing increased the expression of *PmMyD88* at 6, 18, 30 and 36 hpi (Fig. 3B), as well as *PmDorsal* at 6 and 12 hpi (Fig. 3C). Clearly, *PmAP2-β* mediates the Toll signaling pathway during YHV infection.

On the contrary, YHV infection only induced the expression of *PmRelish*, representing the Imd pathway, by threefold at 18 hpi, and *PmAP2-β* silenced shrimp did not show significant changes in *PmRelish* expression during YHV infection, except at 12 hpi (Fig. 3D). It is likely that the Imd pathway may not play an essential role in response to YHV infection.

In addition, the role of the JAK/STAT signaling pathway during YHV infection was investigated by measuring the transcription levels of *PmDOME*, *PmJAK* and *PmSTAT*. Figure 4A showed that YHV-challenged shrimp have a similar expression of *PmDOME*, compared with that in non-infected shrimp. Meanwhile, *PmJAK* was mostly down-regulated during YHV infection (Fig. 4B), while *PmSTAT* expression remained unchanged upon YHV infection, except at 24 and 36 hpi, at which *PmSTAT* was up-regulated around threefold (Fig. 4C). Silencing of *PmAP2-β* increased expression of *PmDOME* in YHV-challenged shrimp by fourfold at 24 hpi (Fig. 4A) and caused an up-regulation of *PmSTAT* by eightfold at 6 hpi and by approximately fourfold at 18, 30 and 36 hpi (Fig. 4C), in comparison with non-challenged shrimp. This result indicated that *PmAP2-β* might be associated with *PmSTAT* activation.

**Effect of *PmAP2-β* silencing on the expression of antimicrobial peptides during YHV infection.** In this work, we investigated the influence of *PmAP2-β* knockdown on the expression of ALF*Pm3*, Crustin*Pm1*, Crustin*Pm7*, PEN3 and PEN5. Figure 5A showed that ALF*Pm3* was highly up-regulated by 16, 15, 30, 24, 3, and 25-fold at 6, 12, 18, 24, 30 and 36 h after YHV infection, respectively. Crustin*Pm1* was increased by threefold at 6 h upon YHV infection (Fig. 5B), while PEN3 was up-regulated at the highest level at 18 hpi (Fig. 5D). In contrast, Crustin*Pm7* and PEN5 seemed to give minimal response to YHV infection (Fig. 5C,E).

Silencing of *PmAP2-β* significantly increased ALF*Pm3* transcripts by 47, 55, 34, 58, 79, 12-fold at 6, 12, 18, 24, 30 and 36 hpi (Fig. 5A). Similarly, knockdown of *PmAP2-β* enhanced Crustin*Pm1* transcription level by 4,
Figure 3. Effect of PmAP2-β silencing on the Toll and the Imd signaling pathways during YHV infection. Shrimp were double injected with either 150 mM NaCl, GFP dsRNA (10 µg/g shrimp) and PmAP2-β dsRNA (10 µg/g shrimp). After YHV injection, shrimp hemocytes were collected at 6, 12, 18, 24, 30, 36 h for qRT-PCR analysis of the transcription levels of PmSpätzle (A), PmMyD88 (B), PmDorsal (C) and PmRelish (D). The data are shown as the mean ± standard deviation. An asterisk represents significant differences from control group (p < 0.05). The experiment was carried out in triplicate.

Figure 4. Effect of PmAP2-β silencing on the JAK/STAT pathways during YHV infection. PmAP2-β knockdown was carried out as described in "Methods". The transcription levels of PmDOME (A), PmJAK (B) and PmSTAT (C) at 6, 12, 18, 24, 30, 36 h after YHV infection were determined by qRT-PCR. The data are shown as the mean ± standard deviation. An asterisk represents significant differences from control group (p < 0.05). The experiment was carried out in triplicate.
5 and threefold at 6, 12 and 24 h post-YHV infection (Fig. 5B). It is worth noting that expression of PEN5 in PmAP2-β silenced shrimp was also increased by 4.5-fold at 6 h after YHV challenge (Fig. 5E), while PmAP2-β silencing did not enhance the expression of CrustinPm1 (≤ twofold) (Fig. 5C,D). Clearly, ALFPm3 and CrustinPm1 play an important role during YHV infection and their expressions were influenced by PmAP2-β.

ALFPm3 and crustinPm1 are responsible for defence against YHV. Roles of ALFPm3 and CrustinPm1 against YHV were further investigated. Either ALFPm3 or CrustinPm1 or both ALFPm3 and CrustinPm1 were knocked down using ALFPm3 dsRNA and/or CrustinPm1 dsRNA of 1 µg per 1 g shrimp as described in "Methods". Figure 6A–C showed that ALFPm3 and CrustinPm1 were successfully knocked down. Silencing of either ALFPm3 or CrustinPm1 alone did not alter shrimp's survival rate upon YHV infection (Fig. 6D). However, knockdown of both ALFPm3 or CrustinPm1 significantly reduced survival percentage at day 2 and 3 post-YHV infection. It is likely that ALFPm3 and CrustinPm1 covered for each other in a defence against YHV.

Discussion
Clathrin-mediated endocytosis plays an essential role in YHV entry into shrimp cells45. In this work, we studied the effects of PmAP2-β silencing on gene expression and shrimp mortality during YHV infection. PmAP2-β is a large subunit 2β of the AP-2 complex, which interacts with clathrin. Previously, PmAP2-β has been characterized and was shown to play a role during WSSV infection1.

In this work, PmAP2-β was continuously up-regulated more than twofold during YHV infection (Fig. 1A). In addition, immunofluorescence showed that the level of PmAP2-β protein was also increased in YHV-challenged hemocyte cells, compared with non-infected cells (Fig. 1B). Figure 2 illustrated that clusters of PmAP2-β located

![Figure 5. Determination of the mRNA transcription levels of antimicrobial peptides under influence of PmAP2-β knockdown during YHV infection. ALFPm3 (A), CrustinPm1 (B), CrustinPm7 (C), PEN3 (D) and PEN5 (E) transcripts of PmAP2-β-silenced shrimp challenged by YHV at 6, 12, 18, 24, 30, 36 h were quantified by qRT-PCR and compared with those in unchallenged PmAP2-β-silenced, YHV-challenged, and YHV-challenged + GFP knockdown shrimp.](image-url)
at the plasma membrane of YHV-infected shrimp cells and the sac structures of PmAP2-β, found in the cytoplasm, may contain the virus inside. Knockdown of PmAP2-β gave rise to a delay of shrimp mortality (Fig. 1C), as well as a reduction in YHV copy number (Fig. 1D). Clearly, silencing of PmAP2-β disrupted YHV propagation. This may be a result of lower number of YHV entering shrimp cells via clathrin-mediated endocytosis or the silencing of PmAP2-β triggering shrimp immune responses.

In Drosophila, Spätzle has been characterized as the cytokine-like molecule that binds to Toll receptor, resulting in signaling cascade through MyD88 and transcription factor Dorsal29,30. In this work, the transcription of PmSpätzle and PmMyD88 was up-regulated during YHV infection (Fig. 3A,B), suggesting that YHV activated the Toll pathway. Silencing PmAP2-β dramatically increased PmSpätzle by 31- and 33-fold at 6 and 12 h after YHV challenge (Fig. 3A) and also enhanced PmMyD88 and PmDorsal expression levels (Fig. 3B,C). In unchallenged shrimp, PmAP2-β knockdown did not affect PmSpätzle, PmMyD88 and PmDorsal expression, however, PmAP2-β silenced shrimp exhibited significantly higher expression of these genes during YHV infection, compared with YHV-challenged normal shrimp. This indicated that PmAP2-β may have an influence on the Toll pathway during YHV infection.

Depletion of PmAP2-β seemed to amplify cellular response of the Toll signaling pathway toward YHV infection. In general, endocytosis mediates receptor signaling by (1) controlling the number of receptors present on the plasma membrane for binding extracellular ligands (2) degradation or recycling of internalized receptors modulates the strength and specificity of signal transmission (3) endosomes play a part in intracellular signaling31–33. During Drosophila embryogenesis, Toll signaling was suggested to occur from the endosome rather than on the plasma membrane34. In P. monodon, silencing of early endosome antigen 1 (EEA1) protein (PmEEA1), involving in early endosome fusion, caused a delay in shrimp mortality due to YHV infection35. Similar results were observed in YHV-challenged shrimp with either PmRab7 or PmRab11 suppression36. These suggested that endosome trafficking plays an important role during YHV infection. It is possible that lack of PmAP2-β may impair clathrin-mediated endocytosis, resulting in alteration of signaling. It was previously reported that clathrin and dynamin-deficient cells showed enhanced activation of canonical NF-κB signaling32.

Regarding the Imd signaling pathway, PmRelish expression did not increase significantly during YHV infection and PmAP2-β-deficiency seemed not to influence PmRelish transcript (Fig. 3D). This implied that the Imd pathway may not substantially contribute to YHV infection and PmAP2-β deficiency did not affect the Imd signaling. Somehow, it was previously reported that PmRelish silencing made the shrimp more susceptible to YHV37.

In Drosophila, the Imd pathway regulates immune genes against Gram-negative bacteria38 and also possesses antiviral function39,40. In Chinese white shrimp Fenneropenaeus chinensis, FcIMD was up-regulated upon WSSV challenge, suggesting that the Imd signaling pathway was involved in antiviral innate immunity of shrimp. It was
reported that knockdown of Relish affected the activity of phenoloxidase (PO) and superoxide dismutase (SOD), and total hemocyte count (THC) after WSSV or *Vibrio alginolyticus* infection in crab *Scylla paramamosain*.

Based on *PmDOME, PmJAK* and *PmSTAT* expression, the JAK/STAT did not promptly respond to YHV infection, at an early stage, when only *PmSTAT* was up-regulated around threefold at 24 and 36 hpi (Fig. 4). However, under the suppression of *PmAP2-β*, the *PmSTAT* transcript significantly increased by eightfold at 6 hpi and by fourfold at 18, 30 and 36 hpi, in response to YHV (Fig. 4C). Dvergne and colleagues reported that, in *Drosophila*, recruitment and trafficking of the clathrin-AP complexes into endocytic vesicles towards the lysosome could enhance the JAK/STAT signaling. In contrast, Vidal and co-workers suggested that endocytic trafficking acts as a negative regulator of JAK/STAT signaling in *Drosophila*. We postulated that knockdown of *PmAP2-β* may disrupt clathrin-dependent endocytosis and signaling from endocytic mechanisms, resulting in an increased expression of *PmSTAT*. It is possible that in *P. monodon*, endocytic mechanisms modulate the JAK/STAT signaling negatively.

Regulation of signaling pathways could alter the expression level of antimicrobial peptides (AMPs). In Kuruma shrimp *Marsupenaeus japonicus*, Gram-positive and Gram-negative bacteria can activate the Toll pathway by their pathogen-associated molecular patterns (PAMPs) directly binding to Toll-like receptors, enhancing the expression of AMPs such as ALF-B1, ALF-C2, Crul-1 and Crul-3. Furthermore, injection of activated *PmSpätzle* enhanced transcription levels of ALF*Pm3*, crustin*Pm1*, crustin*Pm7* and peneauidin3 in black tiger shrimp. The recombinant Spätzle-like protein from Chinese shrimp, *Fenneropenaeus chinensis* could also increase crustin 2 expression in crayfish.

Silingnc of *PmRelish* shrimp suppressed the expression level of peneauidin5, but did not affect ALF*Pm3*, crustin*Pm1* and peneauidin3 expression levels. Knockdown of IMD in *Procambarus clarkii* inhibited the expression of *Crul* and 2, ALF 1 and 2 and *Ly51* in red swamp crayfish challenged with *Vibrio anguillarum*. In *crab S. paramampsain*, Relish knockdown caused a downregulation of immune genes such as JAK, crustin and prophenoloxidase.

Regarding the JAK/STAT, knockdown of suppressor of cytokine signaling 2 (SOCS2) increased ALF-C1, C2 and D1, and Crustin 1 expression levels upon *V. anguillarum* challenge. Meanwhile, injection of recombinant SOCS2 reduced STAT phosphorylation and inhibited STAT translocation into the nucleus, resulting in a decline in the AMP expression.

Since *PmAP2-β* seemed to regulate the signaling cascades, effects of *PmAP2-β* silencing on AMP expression have been investigated. In general, ALFs showed broad antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses, while crustins mainly exhibited antibacterial activity and peneauidins mostly functioned against bacteria and fungi. Previously, a suppression subtractive hybridization (SSH) study reported apparently up-regulated AMPs, including ALF*Pm6* and crustin*Pm1* in response to YHV infection. Figure 5 showed that among five AMPs (ALF*Pm3*, Crustin*Pm1*, Crustin*Pm3*, PEN3 and PEN5), ALF*Pm3* was the most active AMPs against YHV. *PmAP2-β* depleted shrimp showed a significant increase in both ALF*Pm3* and Crustin*Pm1* expressions during YHV infection, compared with normal shrimp challenged with YHV. We postulated that *PmAP2-β* depletion amplified the Toll signaling during YHV infection, resulting in elevated levels of ALF*Pm3* and Crustin*Pm1*. Consistent with this, *PmAP2-β*-deprived shrimp were more resistant to YHV, than normal shrimp (Fig. 2C). In addition, ALF*Pm3* and Crustin*Pm1* silenced shrimp had lower survival rate on days 2 and 3, compared with normal shrimp infected by YHV (Fig. 6D). This indicated that ALF*Pm3* and Crustin*Pm1* are important in defence against YHV. Previous research demonstrated that crustin*Pm1* was found in the granule-containing hemocytes targeted by YHV.

In conclusion, this research suggested that clathrin-mediated endocytosis not only functions as an entry route for YHV but also plays a role in regulating the intracellular signals. *PmAP2-β* depletion stimulated the Toll signaling, resulting in elevated levels of ALF*Pm3* and Crustin*Pm1* during YHV infection. Both ALF*Pm3* and Crustin*Pm1* are essential antimicrobial peptides, acting against YHV.

**Methods.** Healthy black tiger shrimp, *P. monodon*, of about 3.23 ± 0.15 g bodyweight, were from Charoen Pokphand Farm in Chanthaburi Province, Thailand. They were acclimated in laboratory tanks (120 L) at ambient temperature (28 ± 4 °C) and maintained in aerated water with a salinity of 20 ppt for at least 1 week before starting the experiments.

**YHV stock preparation.** YHV stock was prepared as described in previous study. Briefly, hemolymph was drained from YHV-infected moribund shrimp by 1 ml syringe containing an equal volume of modified Alsever solution (MAS: 27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0). Hemocytes were removed by centrifugation at 1000g for 10 min at 4 °C. The supernatant was filtered with 0.45 µm MILLEX-HP filter unit and centrifuged at 30,000g for 30 min at 4 °C. The pellet was washed twice with TN buffer (50 mM Tris–HCl, pH 7.4 and 100 mM NaCl), then, aliquoted and kept at – 80 °C until use. YHV copy number was quantified by qRT-PCR using a specific primer pair for YHV genome (YHV-141-F and YHV-206-R in Table S1).

**Expression of *PmAP2-β* during YHV infection.** Healthy shrimp were separated into two groups, each of which consists of nine individuals, and was injected with either 50 µl of PBS or YHV (500,000 copies). Hemolymph was withdrawn from the abdomen connecting to the first pleopod using a 26-gauge needle and a 1 ml syringe containing an equal volume of ice-cold MAS solution. Each sample contains hemolymph from 3 shrimps (approximately 200 µl of hemolymph per individual). Hemocytes were pelleted by centrifugation at 800g for 10 min at 4 °C. Total RNA was extracted by FavorPrep Tissue Total RNA mini kit (Favogen) and followed by cDNA synthesis using RevertAid First Strand cDNA Synthesis kit (ThermoFisher). *PmAP2-β* transcription level was
survival with the ment was carried out in triplicate. Data were analyzed using GraphPad Prism 6 plot, and presented as percent shrimp at 24 h after PBS or dsRNA injection. The mortality was recorded every 12 hpi up to 8 days. This experiment, a diluted YHV solution containing approximately 10,000 copies per µl was injected into during YHV infection.

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citrate solutions for 2 min, and observed using Transmission Electron Microscope Libra 120 Plus (ZEISS) at the antibody solution at 4 °C overnight and stained with uranyl acetate solution for 5 min, followed by Reynolds lead particle was conjugated to primary AP-2β antibody (Abcam) using InnovaCoat Gold Conjugation kit. The gold placed on a Formvar-supported nickel grid. The grids were incubated with 5% BSA in PBS for 1 h. A 10 nm gold conjugated antibody was diluted 1:50 by 1% BSA in PBS. The grids were incubated with diluted gold conjugated by LR White Embedding Medium (EMS). The embedded gills were cut into ultrathin sections (60–70 nm) and numbers and gill tissues were then collected at 30 hpi and immediately fixed by 4% paraformaldehyde. Fixed tissues: 94 °C for 3 min (denaturation), followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. The two PCR product templates were in vitro transcribed using the T7 RiboMAX System (Promega) to produce two complementary single-stranded RNAs. Then, RQ1 RNase- free DNase was added and incubated at 37 °C for 1 h and the single-stranded RNAs were then purified by standard phenol–chloroform extraction. To generate dsRNA, equal amounts of each of the complementary single-stranded RNAs were mixed, incubated at 70 °C for 10 min, and slowly cooled down at room temperature. The quality and quantity of PmAP2-β dsRNA and GFP dsRNA were analyzed by 1% agarose gel electrophoresis and absorbance at 260 nm, respectively.

To study the effect of PmAP2-β silencing, black tiger shrimp were divided into four groups, each of which consisted of 10 individuals and were injected with either PBS (group 1, control), PBS + YHV (group 2), 10 µg of PmAP2-β dsRNA per 1 g of shrimp + YHV (group 3) and 10 µg of GFP dsRNA per 1 g of shrimp + YHV (group 4). In this experiment, a diluted YHV solution containing approximately 10,000 copies per µl was injected into shrimp at 24 h after PBS or dsRNA injection. The mortality was recorded every 12 hpi up to 8 days. This experiment was carried out in triplicate. Data were analyzed using GraphPad Prism 6 plot, and presented as percent survival with the p values calculated by log-rank test.

Immunofluorescence confocal microscopy. Either diluted YHV stock solution (approximately 10,000 copies per µl) or 150 mM NaCl was injected into shrimp. The hemolymph was collected at 24 h post-injection and mixed in an equal volume of 4% paraformaldehyde in PBS. Hemocytes were collected by centrifugation (800g for 10 min at 4 °C), washed 3 times with PBS and fixed on microscope slides. Hemocytes were incubated with 0.1% Triton X-100 in PBS for 5 min and washed 3 times with PBS. Purified rabbit anti-AP-2β (Abcam) polyclonal IgG antibody in a 1:50 dilution in PBSF (PBS with 1% (v/v) FBS) was used to probe PmAP2-β, followed by Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen), diluted 1:500. YHV was detected by monoclonal IgG antibody specific to gp11652, diluted 1:50 in PBSF, followed by a 1:1000 dilution of Alexa Fluor 568 goat antimouse IgG antibody (Invitrogen). Nuclei were stained with 1:1,000 dilution of Hoechst (ThermoFisher) in PBS. The microscope slides containing the stained and fixed hemocytes were then coated by ProLong Gold (Invitrogen) and kept in the dark at 4 °C until they were observed by a confocal fluorescence microscopy.

Visualization of PmAP2-β by TEM. Shrimp (3–5 g) were injected by YHV of approximately 10,000 copy numbers and gill tissues were then collected at 30 hpi and immediately fixed by 4% paraformaldehyde. Fixed tissues were then washed three times with ice-cold PBS and followed by the manufacturer’s protocol for embedding by LR White Embedding Medium (EMS). The embedded gills were cut into ultrathin sections (60–70 nm) and placed on a Formvar-supported nickel grid. The grids were incubated with 5% BSA in PBS for 1 h. A 10 nm gold particle was conjugated to primary AP-2β antibody (Abcam) using InnovaCoat Gold Conjugation kit. The gold conjugated antibody was diluted 1:50 by 1% BSA in PBS. The grids were incubated with diluted gold conjugated antibody solution at 4 °C overnight and stained with uranyl acetate solution for 5 min, followed by Reynolds lead citrate solutions for 2 min, and observed using Transmission Electron Microscope Libra 120 Plus (ZEISS) at the Microscopy Unit of IBT-UNAM.

Mortality assay of PmAP2-β silencing shrimp upon YHV infection. Double-strand RNA of PmAP2-β and GFP were prepared as described previously7. In brief, the PCR products (PmAP2-β and GFP) were amplified separately by specific primers (Supplementary Information, Table S1) with the following conditions: 94 °C for 3 min (denaturation), followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. The two PCR product templates were in vitro transcribed using the T7 RiboMAX System (Promega) to produce two complementary single-stranded RNAs. Then, RQ1 RNase- free DNase was added and incubated at 37 °C for 1 h and the single-stranded RNAs were then purified by standard phenol–chloroform extraction. To generate dsRNA, equal amounts of each of the complementary single-stranded RNAs were mixed, incubated at 70 °C for 10 min, and slowly cooled down at room temperature. The quality and quantity of PmAP2-β dsRNA and GFP dsRNA were analyzed by 1% agarose gel electrophoresis and absorbance at 260 nm, respectively.

Mortality assay of ALFPm3 and/or CrustinPm1 knockdown shrimp upon YHV infection. The DNA amplicon templates of ALFPm3 and CrustinPm1 were amplified using primers in Supplementary Informa-
tion, Table S1 and ALFPm3 and CrustinPm1 dsRNA synthesis was performed as described above. Either ALFPm3 dsRNA or CrustinPm1 dsRNA was injected at 1 μg per 1 g of shrimp and the hemocytes were collected at 24 hpi. Total RNA and cDNA synthesis were performed as described above; and the level of ALFPm3 and CrustinPm1 transcripts were determined by qRT-PCR. In the mortality experiment, shrimp were divided into 6 groups with 10 shrimp per group as followed, Group 1: PBS (control), Group 2: YHV-challenged, Group 3: YHV-challenged + GFP dsRNA, Group 4: YHV-challenged + ALFPm3 dsRNA, Group 5: YHV-challenged + CrustinPm1 dsRNA, and Group 6: YHV-challenged + ALFPm3/CrustinPm1 dsRNAs. After YHV injection, shrimp mortality was recorded every 12 h up to 4 days. The experiment was performed in triplicate and the data were analyzed using GraphPad Prism 6 and presented as percent survival with the p values calculated by log-rank test.

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Author contributions
T.J. conducted all experiments with assistance from P.L. in shrimp culture, gene silencing experiments and qRT-PCR. P.S., A.T. and K.K. supervised T.J, R.S.-M. and A. O.-L. provided access to TEM and supervised T.J on TEM experiment. K.K. designed the experiments, analyzed data and wrote the manuscript with help from T.J. All authors reviewed the results and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.
