Global profiling of megalocytivirus-induced proteins in tongue sole (Cynoglossus semilaevis) spleen identifies cellular processes essential to viral infection

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ABSTRACT

Megalocytivirus is a DNA virus with a broad host range among farmed fish including tongue sole (Cynoglossus semilaevis). In this study, label-free proteomics was performed to examine protein expression in tongue sole spleen induced by megalocytivirus at 8 and 12 days post infection (dpi). Compared to uninfected control fish, virus-infected fish displayed 315 up-regulated proteins and 111 down-regulated proteins at 8 dpi, and 48 up-regulated proteins and 43 down-regulated proteins at 12 dpi. The expressions of five differentially expressed proteins were confirmed by Western blot. The differentially expressed proteins were enriched in the pathways and processes associated with innate immune response and viral infection. Interference with the expression of two up-regulated proteins of the ubiquitin proteasome system (UPS), i.e. proteasome assembly chaperone 2 and proteasome maturation protein, significantly reduced viral propagation in fish, whereas overexpression of these two proteins significantly enhanced viral propagation. Consistently, inhibition of the functioning of proteasome significantly impaired viral replication in vivo. This study provided the first global protein profile responsive to megalocytivirus in tongue sole, and revealed an essential role of UPS in viral infection.

1. Introduction

Iridoviridae is a family of double-stranded, icosahedral-structured DNA virus that can infect many invertebrates and vertebrates including insects, amphibians, fish, and reptiles (reviewed by Williams, 1996). Currently, Iridoviridae is classified into five genera, three of which, i.e., Ranavirus, Lymphocystivirus and Megalocytivirus, have been isolated from aquaculture species (reviewed by Chinchar et al., 2009). Megalocytivirus is known to cause disease in more than 30 farmed fish, notably mandarin fish (Siniperca chuatsi), large yellow croaker (Pseudosciaena crocea), rock bream (Oplegnathus fasciatus), red sea bream (Pagrus major), sea perch (Lateolabrax japonicas), and members of Pleuronectiformes (Chen et al., 2003; He et al., 2000; Jeong et al., 2006; Kurita and Nakajima, 2012; Shi et al., 2004; Wang et al., 2003; Zhang et al., 2011). Megalocytivirus infection can lead to 30%-100% mortality, thus resulting in severe economic losses for the aquaculture industry (reviewed by Williams et al., 2011).

Viral infection is known to change the expression patterns of a broad range of the host's proteins that lead to alterations in a series of molecular, physiological, and biochemical processes of the host, such as transcription and translation, cell cycle, and immune response (Zheng et al., 2011). Therefore, information on proteomic change in the host following viral invasion is important for studying the mechanism of host-virus interaction. Currently, comparative proteomic analysis has been broadly used for investigating protein expression under various conditions. Label-free quantitative proteomic analysis based on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most powerful method to detect and quantify large amounts of proteins (Zheng et al., 2011). Comparing to conventional proteomic technologies, the advantage of this technique is that it is robust, sensitive, accurate, reproducible, and can detect low-abundance proteins (Sandin et al., 2014). In recent years, this technique has been used to examine proteome changes associated with host responses to many viral pathogens including West Nile virus, hepatitis B and C virus, pea seed-borne mosaic virus, and porcine reproductive and respiratory syndrome virus (Cerna et al., 2017; Jia et al., 2017; Li et al., 2017; Ye et al., 2015; Zhang et al., 2015). Tongue sole (Cynoglossus semilaevis) is a member of
Pleuronectiformes and valued economically in China. It is the first species of Pleuronectiformes with whole genome sequence completed (Chen et al., 2014). In previous studies, we observed that megalocytivirus RBIV-C1 is highly infectious to Pleuronectiformes, including tongue sole (Zhang et al., 2014, 2015; Zhang and Sun, 2015). Given the available genome information, tongue sole is a good model for the investigation of megalocytivirus infection. In this study, we aimed to identify globally the proteins of tongue sole involved in the process of megalocytivirus infection by employing the technique of label-free proteomics. For this purpose, we performed comparative quantitative proteomic analysis of the spleen proteins of tongue sole infected with and without RBIV-C1 at different time points, and examined the role of some of the differently expressed proteins in viral infection.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (115.6 ± 18.5 g and 11.8 ± 2.3 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at laboratory for two weeks in natural light photoperiod before experimental manipulation. During this period, the fish were reared in 40L tanks with 22 °C aerated seawater (oxygent: 9.0–11.0 mg/L) at the following density: less than 30 fish (11.8 ± 2.3 g/fish) per tank, or less than 5 fish (115.6 ± 18.5 g/fish) per tank. Fish were fed daily with commercial dry pellet. Before experiment, fish were randomly sampled for the examination of viral presence as reported previously (Chi and Sun, 2015), and no megalocytivirus was detected. Fish were euthanized with 0.1 g/L of tricaine methanesulfonate (Sigma, St. Louis, USA) before tissue collection to minimize suffering.

2.2. Experimental viral infection and sample preparation

For experimental infection, megalocytivirus RBIV-C1 (Zhang et al., 2011a) was suspended in PBS to 10^7 copies/ml. Tongue sole (average weight 2.2 g) were injected with 0.1 ml RBIV-C1 or PBS (control) and maintained at 24 °C. Fish in each group were administered via intraperitoneal (i.p.) injection with 0.1 ml RBIV-C1 or PBS (control) and maintained at 24 °C. Spleen was taken at even-numbered days (2–16 days) from the fish (three fish/time point) under aseptic conditions. The samples were snap-frozen in liquid nitrogen and stored at 80 °C. Viral copy number in spleen was determined by absolute quantitative real time RT-PCR as reported previously (Zhang and Sun, 2015). The experiment was performed three times.

2.3. Protein extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Spleen samples collected above at 8 days post-infection (dpi) and 12 dpi were homogenized and used for protein extraction as reported previously (Zhang et al., 2013a). The extracted protein supernatant was collected and quantified with the BCA Protein Assay Kit (Bio-Rad, USA). For each sample, 344 μg protein was used for filter-aided sample preparation (FASP), and the protein was digested into peptides as reported previously (Wisniewski et al., 2009). High performance liquid chromatography (HPLC) was performed on Easy-nLC (Thermo Scientific, USA), and approximately 2 μg peptide mixture of each sample was loaded onto a reverse phase trap column, Acclaim PepMap100 C18 (Thermo Scientific, USA) connected to a C18-reversed phase analytical column (Thermo Scientific, USA) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology. The linear gradient was as follows: 0–55% buffer B for 220 min, 55–100% buffer B for 8 min, and then hold in 100% buffer B for 12 min.

The mass spectrometry (MS) experiments were performed with a Q-Exactive mass spectrometer (Thermo Scientific, USA) with analysis time of 240 min. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for higher energy collisional dissociation (HCD) fragmentation. Survey scans were acquired at a resolution of 70,000 at m/z 200 (MS^1) and resolution for HCD spectra was set to 17,500 at m/z 200 (MS^2), and isolation width was 2 m/z. Normalized collision energy was 30 eV and the under ratio was defined as 0.1%.

2.4. Protein identification and differential expression analysis

The MS data were analyzed using MaxQuant software (version 1.3.0.5) (Cox and Mann et al., 2008) (Max Planck Institute of Biochemistry, Germany) against the C. semilaevis database from NCBI (37669 total entries, downloaded on 15/07/16). Parameters were set as follows: the search followed an enzymatic cleavage rule of Trypsin/P with the maximal missed Cleavage sites of two; mass tolerance for MS (first search) and MS/MS were both 20 ppm, and main search tolerance was 6 ppm after recalibration of data; for the identification of peptides and proteins, minimal unique peptides were set to 2, and false discovery rate (FDR) allowed set to 0.01. Protein abundance was calculated on the basis of the normalized spectral protein intensity, label-free quantification (LFQ) intensity. The LFQ intensities of each protein per sample from MaxQuant outputs were loaded into Perseus 1.3 (Max Planck Institute, Germany). Statistical significance was analyzed using Student's t-test with values of p < 0.05, and ratio > 2 or < 0.5 indicating significant differences. For the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, the differentially expressed proteins were mapped to metabolic pathways in KEGG using the KOBAS software (Mao et al., 2005). The mass spectrometry proteomics data have been deposited to ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository (Ma et al., 2018) with the dataset identifier PXD011479.

2.5. Antibody preparation and western blot

To obtain antibodies against proteasome assembly chaperone 2 (PAC2), proteasome maturation protein (Pomp), interferon-inducible double-stranded RNA-dependent protein kinase activator (rPKR), and neutrophil cytosol factor 2 (NCF2), recombinant proteins of PAC2, Pomp, rPKR, and NCF2 were prepared (Fig. S1) as described previously (Zhang et al., 2013a). Polyclonal antibodies against rPAC2, rPomp, rPKR, and rNCF2 were prepared from rats as reported previously (Wang and Sun, 2015). Anti - interferon stimulated protein 15 (ISG15) polyclonal antibody was prepared as reported previously (Wang et al., 2012). Mouse anti -β-actin (ACTB) monoclonal antibody was purchased from Abcam (Cambridge, UK). For Western blot, equal amounts of spleen protein from virus-infected (n = 3) and uninfected (n = 3) tongue sole prepared above were resolved in 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, Cambridge, UK). Western blot was performed as reported previously (Zhang et al., 2013a) using the above antibodies. The primary antibodies were detected with goat anti-rat/mouse IgG-horseradish peroxidase antibody (Abcam, Cambridge, England) 1/2000 dilution in PBS). The membranes were washed three times with PBST (0.05% Tween-20 in PBS), and the antibody was detected with BeyoECL Plus kit (Beyotime, Jiangsu, China) and analyzed with Biorad GelDoc XR (Bio-Rad, USA). The intensities of protein bands were determined by ImageJ program 1.48v (Wayne Rasband National Institutes of Health, USA) the relative integrated density was calculated with β-actin as an internal control (Long et al., 2014).
To select effective small interference RNAs (siRNAs) for E3 ubiquitin-protein ligase herc6 (E3-L6), E3 ubiquitin-protein ligase herc4 (E3-L4), PAC2 and Pomp, three 2'-OMe-modified siRNAs for each gene were designed and synthesized by Genepharma (Shanghai, China). As a negative control, nontargeted control siRNA (siCR) was synthesized and presented as means ± SEM.

2.6. Knockdown of PAC2 and Pomp and the effect on viral infection

To examine the effect of E3-L6, E3-L4, PAC2, and Pomp knockdown on viral infection, tongue sole were infected with megalocytivirus RBIV-C1, and viral load in the spleen of the fish was monitored at 2–16 days after infection. Data are the means of three independent experiments and presented as means ± SEM.

2.7. PAC2 and Pomp overexpressions and their effects on viral infection

To construct the plasmids pCN3-PAC2 (pPAC2) and pCN3-Pomp (pPomp) that express His-tagged PAC2 and Pomp, the coding sequences of PAC2 and Pomp were amplified by PCR with primer pairs PAC2-F/PAC2-R and Pomp-F/Pomp-R, respectively (Table S1). The PCR products were ligated with the T-A cloning vector pEASY-Simple-T (Tiangen, Beijing, China), and the recombinant plasmids were digested with EcoRV to retrieve the PAC2 and Pomp fragments, which were inserted into pCN3 (Li et al., 2015) at the EcoRV site, resulting in pPAC2 and pPomp. Endotoxin-free plasmid pPAC2, pPomp and pCN3 were prepared using Endo-Free plasmid Kit (Tiangen, Beijing, China). Western blot was performed as described above. To examine the effect of PAC2 and Pomp overexpression on viral infection, fish were injected with pPAC2, pPomp, pCN3 or PBS as above, and at 3 days post-plasmid administration, total proteins were extracted with Total Protein Extraction Kit (Sangon Biotech, Shanghai, China). Western blot was performed as described above. To examine the effect of PAC2 and Pomp overexpression on viral infection, fish were injected with pPAC2, pPomp, pCN3 or PBS as above, and at 3 days post-plasmid administration, the fish were infected with RBIV-C1 as above. At 3 dpi, 5 dpi, and 7 dpi, viral load in spleen was determined as above. The experiment was performed three times.

2.8. Inhibition of proteasome and its effect on viral infection

Proteasome inhibitor MG132 (Sigma, St. Louis, USA) was dissolved in dimethyl sulfoxide (DMSO) to 1 mg/ml, which was further diluted in PBS to 12 μg/ml. As a control to MG132 solution, DMSO was similarly diluted in PBS without the inhibitor. Tongue sole (average 11.8 g) were divided randomly into five groups (9 fish/group) and injected i.p. with 100 μg of each of the siRNAs described above; the control group of fish was injected with PBS. At 2 dpi, total RNA was extracted from each of the five fish with E.Z.N.A Total RNA Kit (Omega Bio-tek, Doraville, GA, USA) and treated with RNase-free DNase I (Omega Bio-tek) according to manufacturer’s instructions. The quality and integrity of the RNA were examined by determining 260/280 absorbance ratio using NanoDrop 2000 (Thermo Scientific, USA) and by gel electrophoresis. One microgram of total RNA was used for cDNA synthesis with ReverAid™ reverse transcriptase (MBI Fermentas, Canada) according to manufacturer’s instructions. The expression level of E3-L6, E3-L4, PAC2 and Pomp was determined by qRT-PCR in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with SYBR Premix Ex Taq Kit (Takara, Dalian, China) as described previously (Zhang et al., 2016) with the primer pairs E3-L6-RTF/E3-L6-RTR, E3-L4-RTF/E3-L4-RTR, PAC2-RTF/PAC2-RTR, and Pomp-RTF/Pomp-RTR, respectively (Table S1). The relative mRNA expression level of each gene was calculated using β-actin as an internal control (Long et al., 2014). PCR efficiency (E) and correlation coefficient (R2) were determined as reported previously, with negative control without cDNA included in each assay (Zhang et al., 2013b). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected (Fig. S2). For each gene, qRT-PCR was performed five times with RNA from five different fish, and in each time of PCR, the reaction was run in triplicate. The siRNAs with the strongest inhibitory effect on E3-L6, E3-L4, PAC2 and Pomp expression were re-named siE3-L6, siE3-L4, siPAC2 and siPomp, respectively, and their sequences are shown in Table 2. This screening experiment was performed three times.

To examine the effect of E3-L6, E3-L4, PAC2, and Pomp knockdown on viral infection, tongue sole (average 11.8 g) were administered with siE3-L6, siE3-L4, siPAC2, siPomp, siCR, or PBS as above. At 1 day post siRNA administration, the fish were infected with 0.1 ml RBIV-C1 (10⁸ copies/ml) as above. At 2 dpi, the fish were re-administered with siE3-L6, siE3-L4, siPAC2, siPomp, siCR, or PBS as above. At 3 dpi and 5 dpi, spleen was taken from the fish under aseptic conditions and examined for viral load as described above. The experiment was performed three times.

2.9. Statistical analysis

All experiments were performed three times, and statistical analyses were performed using analysis of variance (ANOVA) in SPSS 17.0 package (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as P < 0.05.

3. Results

3.1. Infection and replication of megalocytivirus RBIV-C1 in tongue sole

Following infection of tongue sole with RBIV-C1, viral dissemination and propagation in spleen was monitored at different days. The viral load was 10³.6 to 10³.9 copies/mg at 2 to 4 dpi, increased steadily with time to 10⁶.0 copies/mg by 10 dpi, and then decreased to 10³.8 copies/mg by 16 dpi (Fig. 1). No virus was detected in the control group of fish. Based on these observations, 8 dpi, the point where viral replication was robust, and 12 dpi, the point where viral number declined, were selected for proteomic analysis.
3.2. Label-free proteomics analysis of virus-induced protein expression in tongue sole

3.2.1. Overview of the protein expression profile

Label-free proteomic analysis was conducted to examine the protein expression profile in the spleen of virus-infected (n = 3) and uninfected (n = 3) tongue sole at 8 dpi and 12 dpi. The quantitative expression ratio of infected fish/control fish with a value greater than 2.0 and \(P < 0.05\) was considered up-regulated, while the quantitative expression ratio of infected/control fish with a value quantitative ratio of less than 0.5 and \(P < 0.05\) was considered down-regulated. At 8 dpi, 24,727 unique peptides corresponding to 2671 distinct proteins were identified, while at 12 dpi, 25,173 unique peptides corresponding to 2753 distinct proteins were identified. At 8 dpi, 315 proteins were up-regulated and 111 proteins were down-regulated; of the differentially expressed proteins (DEPs), 193 were only detected in virus-infected group, and 34 were only detected in control group (Fig. 2A; File S1). At 12 dpi, 48 proteins were up-regulated and 43 proteins were down-regulated; 41 DEPs were only detected in virus-infected group, and 37 DEPs were only detected in control group (Fig. 2A; File S2). Twenty-one DEPs were shared by 8 dpi and 12 dpi groups (Fig. 2B; Table S2).

3.2.2. Functional annotation of the DEPs

Function annotation revealed that, of the DEPs at 8 dpi, 22 were with immune functions, including 18 up-regulated proteins and 4 down-regulated proteins. The up-regulated proteins were interferon regulatory factor (IRF) (2 proteins), interferon inducible proteins (3 proteins), heat shock protein (4 proteins), MHC class I and II, lysozyme (3 proteins), complement c1q, lectin (2 proteins), cytokine receptor-like factor 3, and interleukin enhancer-binding factor 3 (Table 1). The down-regulated proteins with immune-related functional annotations were tripartite motif-containing protein (2 proteins), complement factor d-like, and NCF2 (Table 1). Thirty-nine DEPs at 8 dpi were associated with viral infection, including 31 up-regulated proteins and 8 down-regulated proteins; the up-regulated proteins were ubiquitin proteasome system (UPS) related proteins (10 proteins), mannose receptor (3 proteins), cathepsin (Z and S), cytoskeletal proteins (2 proteins), importin (6 proteins), transcription and translation factors (7 proteins), and apoptosis inhibitor 5. The down-regulated proteins associated with viral infection were E3 ubiquitin-protein ligase (2 proteins), cytoskeleton (3 proteins), and integrin (3 proteins) (Table 1). Of the DEPs at 12 dpi, four were immune-associated, including 3 up-regulated proteins (heat shock 70, C-type lectin, and MHC class II

![Fig. 2. Differentially expressed proteins identified by Label-Free quantitative proteomics. (A) Summary of up-regulated and down-regulated proteins at 8 and 12 days post infection (dpi). (B) Venn diagram showing the numbers of differentially expressed proteins at 8 dpi and 12 dpi.](image)

Table 1
Summary of differentially expressed proteins related to immunity and viral infection.

| Protein name                                                                 | GenBank Accession No. | Unique peptides | Sequence coverage (%) | Mol. weight [kDa] | Up/Down Regulated | dpi |
|------------------------------------------------------------------------------|-----------------------|-----------------|-----------------------|------------------|--------------------|-----|
| Interferon regulatory factor 3                                               | XP_008312881          | 8               | 32.2                  | 47.1             | Up                 | 8   |
| Interferon regulatory factor 4-like isoform x1                              | XP_016891299          | 2               | 9.5                   | 45.1             | Up                 | 8   |
| Interferon inducible mx protein                                             | XP_008318598          | 39              | 68.8                  | 70.5             | Up                 | 8   |
| Interferon-induced protein 44-like isoform x1                               | XP_016898236          | 17              | 62.8                  | 35.3             | Up                 | 8   |
| Interferon stimulated protein 15 (ISG15)                                    | NP_001287935          | 9               | 79.6                  | 18.3             | Up                 | 8   |
| Interferon-inducible double-stranded ma-dependent protein kinase activator a (PRR) | XP_008326104         | 5               | 23.2                  | 32.9             | Down               | 12  |
| Tripartite motif-containing protein 35-like                                  | XP_016890245          | 3               | 8.9                   | 53.416           | Down               | 8   |
| Tripartite motif-containing protein 16-like                                  | XP_008331037          | 4               | 7.2                   | 60.926           | Down               | 8   |
| Heat shock protein hsp 90-beta                                              | XP_008311248          | 23              | 53                    | 83.3             | Up                 | 8   |
| Heat shock protein hsp 90-alpha                                             | XP_008311363          | 5               | 26                    | 84.0             | Up                 | 8   |
| Heat shock protein 75 mitochondrial                                         | XP_008328085          | 5               | 8.5                   | 82.0             | Up                 | 8   |
| Heat shock 70 kda protein 14                                                | XP_008314207          | 4               | 11.8                  | 55.1             | Up, Up             | 8, 12|
| MHC class i a chain                                                         | AC88824               | 10              | 40.5                  | 40.8             | Up                 | 8   |
| MHC class ii antigen partial                                               | ADG45981              | 2               | 49.5                  | 12.4             | Up                 | 8   |
| MHC class ii a antigen                                                      | AC205677              | 2               | 20.2                  | 25.8             | Up                 | 12  |
| Lysozyme g-like isoform x2                                                 | XP_016887901          | 8               | 65.5                  | 21.4             | Up                 | 8   |
| Lysozyme g-type                                                            | NP_001281118          | 4               | 26.5                  | 23.7             | Up                 | 8   |
| Lysozyme C-type                                                            | XP_008322585          | 5               | 41.7                  | 16.2             | Up                 | 8   |
| Complement factor d-like                                                    | XP_008314450          | 6               | 37.7                  | 30.0             | Down               | 8   |
| Complement c1q-like protein 2                                               | XP_008328268          | 3               | 24.9                  | 25.2             | Up                 | 8   |
| C-type lectin domain family 10 member                                       | XP_008322850          | 2               | 6.5                   | 31.6             | Up                 | 12  |

(continued on next page)
Galectin-8-like isoform x1
Interleukin enhancer-binding factor 3
Neutrophil cytokosol factor 2 (NGF2)
Cytokine receptor-like factor 3

Proteins related to viral infection
E3 ubiquitin-protein ligase trim9-like isoform x1
E3 ubiquitin-protein ligase lrsam1
E3 ubiquitin-protein ligase trip12 isoform x1
E3 ubiquitin-protein ligase herc6 isoform x2 (E3-L6)
E3 ubiquitin-protein ligase herc4
Ubiquitin carboxyl-terminal hydrolase 4 (E3-L4)
Ubiquitin-like-conjugating enzyme atg3 isoform x2
Ubiquitin-conjugating enzyme E2 I3-like
Proteasome assembly chaperone 2 (PAC2)
Proteasome maturation protein (Pomp)
Integrin beta-3-like
Integrin alpha-m-like
Integrin alpha-v-like
Macrophage mannose receptor 1-like
Macrophage mannose receptor 1
Mannose-specific lectin-like
Cathespin z
Cathespin s
Type ii cytoskeletal 8-like
Echinoderm microtubule-associated 3
Cytoskeleton-associated protein 4
Type i cytoskeletal 18
Apoptosis inhibitor 5

* Number of peptides detected.
* Detected peptide coverage of protein sequence in %.
* Proteins identified in RBIV-C1 infected fish compared to control fish.
* Days post infection.

Table 1 (continued)

| Protein name                                      | GenBank Access No. | Unique peptides | Sequence coverage (%) | Mol. weight [kDa] | Up/Down Regulated | dpi |
|--------------------------------------------------|--------------------|-----------------|-----------------------|------------------|-------------------|-----|
| Galectin-8-like isoform x1                        | XP_008311902       | 2               | 7.1                   | 34.3             | Up                | 8   |
| Interleukin enhancer-binding factor 3            | XP_008328908       | 6               | 11.6                  | 92.5             | Up                | 8   |
| Neutrophil cytokosol factor 2 (NGF2)             | XP_016886943       | 11              | 28.3                  | 56.0             | Down              |     |
| Cytokine receptor-like factor 3                  | XP_016895705       | 9               | 30.7                  | 43.8             | Up                | 8   |
| E3 ubiquitin-protein ligase trim9-like isoform x1| XP_016886682       | 4               | 6.4                   | 101.0            | Up                | 8   |
| E3 ubiquitin-protein ligase lrsam1               | XP_016894516       | 6               | 9.2                   | 84.1             | Down              | 8   |
| E3 ubiquitin-protein ligase trip12 isoform x1    | XP_016887407       | 6               | 4.4                   | 224.8            | Up                | 8   |
| E3 ubiquitin-protein ligase herc6 isoform x2     | XP_016887709       | 23              | 32.4                  | 111.7            | Up                | 8   |
| E3 ubiquitin-protein ligase rho3                 | XP_008306043       | 4               | 4.2                   | 123.6            | Down              | 8   |
| E3 ubiquitin-protein ligase herc4                | XP_008309258       | 5               | 6.8                   | 112.1            | Up, Up            | 8, 12|
| Ubiquitin carboxyl-terminal hydrolase 4          | XP_008318167       | 5               | 9.1                   | 113.0            | Up                | 8   |
| Ubiquitin-like-conjugating enzyme atg3 isoform x2| XP_008323262       | 3               | 12.9                  | 36.2             | Up, Down          | 8, 12|
| Ubiquitin-4 isoform x2                           | XP_008329855       | 3               | 6.6                   | 65.3             | Up                | 8   |
| Ubiquitin-conjugating enzyme E2 I3-like          | XP_008323810       | 7               | 68.2                  | 17.8             | Up                | 8   |
| Proteasome assembly chaperone 2 (PAC2)           | XP_008329732       | 8               | 39                    | 29.3             | Up                | 8   |
| Proteasome maturation protein (Pomp)             | XP_008305862       | 2               | 20.6                  | 15.9             | Up                | 8   |
| Integrin beta-3-like                             | XP_016896081       | 16              | 25.5                  | 92.6             | Down              | 8   |
| Integrin alpha-m-like                            | XP_008312834       | 32              | 39.5                  | 125.7            | Down              | 8   |
| Integrin alpha-v-like                            | XP_008327192       | 21              | 29                    | 114.2            | Down              | 8   |
| Macrophage mannose receptor 1-like               | XP_008332739       | 18              | 18.9                  | 160.1            | Up                | 8   |
| Macrophage mannose receptor 1                    | XP_008306513       | 10              | 10.9                  | 164.9            | Up                | 8   |
| Mannose-specific lectin-like                     | XP_008316311       | 4               | 55.8                  | 13.0             | Up                | 8   |
| Cathespin z                                      | XP_008318331       | 9               | 52.3                  | 33.8             | Up                | 8   |
| Cathespin s                                      | XP_008322034       | 12              | 50.3                  | 37.5             | Up                | 8   |
| Type ii cytoskeletal 8-like                      | XP_016892340       | 11              | 32                    | 55.8             | Down              | 8   |
| Echinoderm microtubule-associated 3              | XP_008326339       | 4               | 7.6                   | 111.0            | Up                | 8   |
| Cytoskeleton-associated protein 4                | XP_008313795       | 6               | 14.9                  | 55.2             | Up                | 8   |
| Type i cytoskeletal 18                           | XP_008318457       | 34              | 82.9                  | 45.2             | Down              | 8   |
| Tubulin beta chain-like                          | XP_008313038       | 20              | 57.2                  | 53.2             | Down              | 8   |
| Tubulin beta-4b chain                            | XP_008335861       | 2               | 58.3                  | 49.8             | Down              | 12  |
| Importin-5                                       | XP_008322931       | 27              | 38.6                  | 122.4            | Up                | 8   |
| Importin-9                                       | XP_008319310       | 10              | 14.5                  | 115.6            | Up                | 8   |
| Importin-7                                       | XP_008308980       | 19              | 26.2                  | 118.9            | Up                | 8   |
| Importin-11 isoform x1                           | XP_008334270       | 6               | 11.1                  | 111.8            | Up                | 8   |
| Importin subunit alpha-3                         | XP_008307947       | 5               | 27.7                  | 57.7             | Up                | 8   |
| Importin subunit alpha-4                         | XP_008326400       | 6               | 26.3                  | 57.7             | Up                | 8   |
| Low quality protein: transcription factor p55    | XP_008317791       | 3               | 7                     | 67.3             | Up                | 8   |
| Nuclear transcription factor y subunit gamma isoform x3 | XP_008329866 | 3               | 9.6                   | 38.1             | Up                | 8   |
| Eukaryotic translation initiation factor 4       | XP_016897922       | 9               | 9                     | 188.6            | Up                | 8   |
| DNA-directed rna polymerase ii subunit rbp4      | XP_008313705       | 4               | 42.4                  | 16.3             | Up                | 8   |
| Eukaryotic translation initiation factor 6       | XP_008319381       | 5               | 40.2                  | 26.6             | Up                | 8   |
| Pre-mrna-processing factor 39-like               | XP_008325063       | 4               | 10.2                  | 82.6             | Up                | 8   |
| Eukaryotic translation initiation factor 5b      | XP_008323571       | 12              | 14.7                  | 135.9            | Up                | 8   |
| Apoptosis inhibitor 5                            | XP_008309710       | 16              | 39.5                  | 59.0             | Up                | 8   |

The expression levels of five DEPs (PAC2, Pomp, ISG15, NCF2, and PKR) were examined by Western blot with antibodies against the proteins related to immune defense and viral infection, which included p53 signaling pathway, ubiquitin mediated proteolysis, phagosome, HTLV-1 infection, Epstein-Barr virus infection, and apoptosis (Fig. 3A and File S3). At 12 dpi, only 91 DEPs were enriched in three KEGG pathways, i.e., gap junction, cytokine-cytokine receptor interaction, and C5 branced dibasic acid metabolism (Fig. 3B and File S3).
respective proteins in the recombinant form prepared from *E. coli*. The results showed that consistent with proteomic analysis, PAC2, Pomp, and ISG15 were significantly up-regulated in virus-infected fish at 8 dpi, with a fold change of 2.96, 3.17 and 18.97, respectively, while NCF2 and PKR were significantly down-regulated in virus-infected fish at 8 dpi (0.33 fold) and 12 dpi (0.24 fold), respectively (Fig. 4).

3.4. Effect of E3-L6, E3-L4, PAC2, and Pomp on viral infection

3.4.1. Knockdown of E3-L6, E3-L4, PAC2, and Pomp

Four genes, i.e. E3-L6, E3-L4, PAC2, and Pomp, involved in ubiquitin proteasome system were investigated for their potential essentialness to viral infection. For this purpose, gene knockdown was performed by siRNAs targeting E3-L6, E3-L4, PAC2, and Pomp (siE3-L6, siE3-L4, siPAC2, and siPomp, respectively). Subsequent qRT-PCR analysis showed that at 2 d post siRNA administration, the expression levels of E3-L6, E3-L4, PAC2, and Pomp in the spleen of the fish administered with their respective siRNAs were significantly (*P* < 0.01) reduced to the levels of 64.7%, 60.4%, 55.8%, and 54.1%, respectively, of that in the control fish (Fig. S3). In contrast, the expression levels of the four genes in fish administered with control siRNA (siCR) were similar to that in the control fish (Fig. S3).

3.4.2. Effect of E3-L6, E3-L4, PAC2, and Pomp knockdown on viral infection

To examine the effect of E3-L6, E3-L4, PAC2, and Pomp knockdown on viral replication, the fish were infected with RBIV-C1, and viral load in spleen was determined at 3 dpi, 5 dpi, and 7 dpi. The results showed that knockdown of PAC2 by siPAC2 significantly reduced the viral number by 2.2-fold, 13.3-fold and 12.5-fold at 3 dpi, 5 dpi, and 7 dpi, respectively (Fig. 5). Knockdown of Pomp by siPomp significantly reduced the viral number at 5 dpi and 7 dpi by 8.1-fold and 5.3-fold, respectively (Fig. 5). In contrast, knockdown of E3-L6 and E3-L4 by siE3-L6 and siE3-L4, respectively, had no apparent effect on viral replication (data not shown).

3.5. Overexpression of PAC2 and Pomp in tongue sole and its effect on viral infection

To further examine the importance of PAC2 and Pomp in viral infection, the two proteins were over-expressed in tongue sole by administering into the fish the plasmids pPAC2 and pPomp, which express His-tagged PAC2 and Pomp, respectively. Western blot showed that at 3 d post-plasmid administration, His-tagged PAC2 and Pomp proteins were detected in the spleen of the fish administered with pPAC2 and pPomp, respectively, but not in the control fish or the fish administered with the control plasmid pCN3 (Fig. S4). To examine the effect of PAC2 and Pomp overexpression on viral infection, the fish were challenged with RBIV-C1 at 3 days post-plasmid administration, and viral replication in spleen was determined at 3 dpi, 5 dpi, and 7 dpi. The results showed that compared to control fish, the viral copies in pPAC2-administered fish were 6.1-, 5.7-, and 3.5-fold higher at 3 dpi, 5 dpi, and 7 dpi, respectively, which were significantly higher than that in the control fish. Compared to the control fish, the viral copies in pPomp-administered fish were 3.7- and 2.6-fold higher at 3 dpi and 5 dpi, respectively (Fig. 6), which were significantly higher than that in the control fish.

3.6. Effect of proteasome inhibitor on viral infection

To further examine the potential importance of proteasome in viral infection, proteasome in tongue sole was inhibited by MG132 before RBIV-C1 challenge. Subsequent analysis showed that viral copies in fish treated with MG132 were significantly lower than that in the control fish, with a fold difference of 5.7 and 12.2 at 5 dpi and 7 dpi,
respectively (Fig. 7).

4. Discussion

In this study, we examined the protein expression profile induced by megalocytivirus RBIV-C1 in tongue sole. A total of 426 DEPs were identified at 8 dpi when viral replication in fish was robust, while only 91 DEPs were identified at 12 dpi when viral replication began to decline, suggesting a much more fierce battle occurring between the host and virus at the middle infection stage than at the late infection stage when the host defense system began to dominate.

Of the DEPs, some are apparently associated with innate immune defense, which provides the first line of defense against invading pathogens. In response to virus infection, the innate immune pathways are activated and a variety of cytokines and interferons (IFNs) are released (Mandelboim et al., 2001). IFNs have long been known to play an essential role in antiviral immunity (Gonzalez-Navaia et al., 2012). In our study, no IFNs proteins were detected among RBIV-C1-induced DEPs, but DEPs in the nature of IRFs, which are regulators of IFN transcription, were identified. Of the identified IRFs, previous reports showed that IRF3 is an important factor involved in modulating the transcription of type I IFN and IFN-stimulated genes (ISGs), and IRF4 is expressed predominantly in immune cells and acts as a critical determinant for the regulation of downstream antiviral cytokines such as IFN-γ (Lu et al., 2006). In fish, it has been shown that the expression of IRF3 gene could be stimulated by nervous necrosis virus and poly (I:C) (Laghari et al., 2018; Labella et al., 2018), and IRF4 gene could be stimulated by rock bream iridovirus and poly (I:C) (Bathige et al., 2012; Krasnov et al., 2013). In our study, we found that significantly up-regulated IRF3 and IRF4 proteins occurred at 8 dpi, suggesting a potential role of IRFs and the IFN system in the early immune defense against megalocytivirus.

Besides IRFs, several differentially expressed ISGs were detected in RBIV-C1-infected fish. ISGs are initiated by IFNs and act as primary...
factors in controlling the replication and spread of virus in vivo (Morales and Lenschow, 2013). In fish, some ISGs, such as viperin, ISG15, ISG56, Mx, tripartite motif (TRIM) 8, TRIM39, and PKR (interferon-inducible double-stranded rna-dependent protein kinase activator a), have been shown to be antiviral effectors capable of limiting viral replication (reviewed by Poynter and DeWitte-Orr, 2016). In this study, we detected three ISG proteins, i.e., Mx, ISG44, and ISG15, up-regulated by RBIV-C1. Mx is one of the most studied fish ISGs, which is induced by type I IFN and exhibits antiviral properties in a wide range of fish, notably infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon (Larsen et al., 2004), hemorrhagic septicemia virus (VHSV) infected Japanese flounder (Caijang et al., 2003), nodavirus infected grouper (Chen et al., 2008) and barramundi (Wu et al., 2010). ISG15 is a small, ubiquitin-like molecule with numerous antiviral functions including inhibition of virus release and ISGylation of both viral and host proteins, and it also shows broad antiviral activities against a number of fish viruses including grouper nervous necrosis virus (GNNV), infectious hematopoietic necrosis virus (IHNV), VHSV, and RBIV-C1 (Huang et al., 2013; Langevin et al., 2013; Wang et al., 2012). The up-regulation of ISGs detected in our study is consistent with these previous observations and suggests that, like in other teleosts, ISGs likely play an important role in the antiviral immunity of tongue sole.

In addition to the up-regulated ISG proteins, three down-regulated ISG proteins, i.e., TRIM16-like, TRIM35, and PKR, were identified in RBIV-C1-infected tongue sole. TRIM family is an emerging group of antiviral ISGs, and members of this family are believed to interfere with viral replication (reviewed by Poynter and DeWitte-Orr, 2016). In this study, we detected three down-regulated ISG proteins, i.e., Mx, ISG44, and ISG15, up-regulated by RBIV-C1. Mx is one of the most studied fish ISGs, which is induced by type I IFN and exhibits antiviral properties in a wide range of fish, notably infectious pancreatic necrosis virus (IPNV) infected Atlantic salmon (Larsen et al., 2004), hemorrhagic septicemia virus (VHSV) infected Japanese flounder (Caijang et al., 2003), nodavirus infected grouper (Chen et al., 2008) and barramundi (Wu et al., 2010). ISG15 is a small, ubiquitin-like molecule with numerous antiviral functions including inhibition of virus release and ISGylation of both viral and host proteins, and it also shows broad antiviral activities against a number of fish viruses including grouper nervous necrosis virus (GNNV), infectious hematopoietic necrosis virus (IHNV), VHSV, and RBIV-C1 (Huang et al., 2013; Langevin et al., 2013; Wang et al., 2012). The up-regulation of ISGs detected in our study is consistent with these previous observations and suggests that, like in other teleosts, ISGs likely play an important role in the antiviral immunity of tongue sole.

In our study, no differentially expressed cathepsin B and L proteins were identified in RBIV-C1-infected fish, however, up-regulated cathepsin Z and S were detected, suggesting that more types of cathepsins are involved in viral infection in fish.

In order for the transcription of the viral genes in host cells, the viral genome has to be transported into the nucleus. Nuclear import of the viral genome is a highly selective process that requires cargo proteins containing nuclear localization signals and importin that mediates nuclear import (Wente and Rout, 2010). Consistently, in RBIV-C1-infected fish, we found 6 up-regulated importins at 8 dpi. Once the viral genome is imported into the nucleus, transcription and translation of the viral genes take place, during which, some host cellular molecules, such as transcription factors and translation initiation factors, are utilized by viruses (Sengupta et al., 2017; Yuan et al., 2017; Zhou et al., 2015). Similarly, in our study, we observed up-regulated transcription factors and translation initiation factor 4 and 6 proteins in RBIV-C1-infected tongue sole. Besides these factors, we also detected pre-mRNA-processing factor and DNA-directed RNA polymerase II proteins, suggesting employment of host molecules for the transcription and translation of viral genome.

In eukaryotic cells, the ubiquitin proteasome system (UPS) is the major intracellular protein degradation and regulation pathway that plays a key role in diverse cellular processes, including apoptosis, cell cycle, signal transduction, host immune response, endocytosis, and signal transduction (Foo and Chee, 2015). Evidences show that UPS is important for the uncoating, nucleus entry, genome transcription and translation, and the budding process of many mammalian viruses.
including herpesvirus, poxvirus, hepatitisvirus, adenovirus, influenza viruses, retrovirus, coronavirus, paramyxovirus, picornavirus and rotavirus (reviewed by Glickman and Ciechanover, 2002; Calistri et al., 2014). In fish, the function of UPS is largely unknown. However, UPS genes were reported to be regulated in IHNV-infected rainbow trout and ISKNV-infected mandarin fish (He et al., 2006; Liang and Cui, 1982). A recent study showed that mandarin fish UPS components can be used by ISKNV, suggesting a potential involvement of UPS in virus-host interaction (Huang et al., 2017). Likewise, in our study, 12 UPS related proteins were regulated by RBIV-C1, including E2, E3 ubiquitin, PAC2, and Pomp. Of these proteins, PAC2 and Pomp are important to the process of proteasome assembly and maturation (reviewed by Budenholzer et al., 2017). In line with this fact, we found that overexpression of PAC2 and Pomp in tongue sole significantly enhanced viral replication, while knockdown of these genes or inhibiting the function of UPS significantly inhibited viral replication. These results suggest that UPS is essential to viral infection, and this property of UPS may have been utilized by RBIV-C1 to facilitate viral propagation by up-regulating UPS expression during infection. Since E3 ubiquitin ligases, which determine the substrate specificity of ubiquitination, exist in hundreds of different forms (Gao et al., 2013), it is likely that the E3 ubiquitin ligases examined in our study are not the major ones targeted by RBIV-C1, or the functions of these two E3 ligases are replaceable by other members of E3 ligases.

In conclusion, in this study we revealed for the first time the global protein profile of tongue sole induced by megalocytivirus. Besides the proteins associated with immune response and various stages of viral infection, the ubiquitin proteasome system was found to play a significant role in promoting the replication of megalocytivirus and may consequently serve as a target for viral manipulation of the host’s immune defense. These results add new insights into fish antiviral immunity as well as the immune evasion strategy of megalocytivirus.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdb.2018.11.006.

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