Supplementary Materials for

**Temperature-dependent IL6-STAT3-HSP90 signaling mediates viral entry**

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This PDF file includes:

Materials and Methods

Figure Legends S1-S7

Figure S1 to S7

Supporting videos legends 1-2

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animal ethic statements**

All procedures with grass carp, koi fish and zebrafish used in experiments were approved by the Ethics Committee of Hunan agriculture University and the methods were carried out following the approved guidelines of animal ethic statements.

**Fish**

Grass carp about 8-10 cm in length and koi fish about 6-8 cm in length were utilized for viral infection or temperature stress experiments. Adult zebrafish were raised and kept in a recirculating system at 28 °C with 14 h light/h dark cycle.

**Cells**

Fish cell line such as CIK (*Ctenopharyngodon idellus* kidney cells), CCB (Common carp brain cells), EPC (Epithelioma Papulosum Cyprini cells), GSM (Chinese giant salamander muscle cells) were maintained in medium 199 (M199) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 ug/ml) at 28°C in carbon dioxide-free atmosphere. Mammalian cell line such as HEK293T (human embryonic kidney cells), NIH 3T3 (3T3-Swiss albino cells), VERO cells, PK-15 (porcine kidney epithelial cells), and MEF (mouse fibroblast cells) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 ug/ml) at 37 °C in a humidified 5% CO2 atmosphere. Insect cell line SF9 (Spodoptera frugiperda cells) were cultured in SF-900™ II SFM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 ug/ml) at 28°C in carbon dioxide-free atmosphere.

**Virus preparation**

Grass carp reovirus (GCRV), cyprinid herpesvirus-3 (CyHV-3), spring viremia of carp virus (SVCV), Chinese giant salamander iridovirus (GSIV), Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV), porcine epidemic diarrhea virus (PEDV), murine gamma herpesvirus 68 (MHV68), herpes simplex virus 1 (HSV-1) were amplified in CIK, EPC, GSM, SF2, PK, MEF, and VERO cells accordingly. The supernatant containing infectious virion particles was collected and centrifuged at 1000 g for 10 min. Virus was concentrated by ultracentrifugation at 32,500 g for 2 h, if necessary.

**METHOD DETAILS**

**Temperature stress experiment**

Grass carp about 8-10 cm in length were temporarily raised in a tank of 18 °C water temperature for 1 week of adaptation. After then fish were transferred to a tank of 28 °C water temperature. Tissue samples were collected at different time point for RT-PCR analysis. CIK or GCO cells were cultured at 18 °C or 28 °C cell incubators for 48 h for adaptation, GCRV infection and temperature stress switching experiments were then conducted from 28 °C to 18 °C or 18 °C to 28 °C, respectively. Cell samples at different time points were collected to analyze the relative gene expression and viral genome replication.

**DNA transfection**

All the plasmid transfections in cells were performed using polyethylenimine (PEI) following the manufacturer’s protocol. Cells were seeded into 6-well plates to the density of 50-70% confluency. Serum media were removed from cells and replaced with serum-free DMEM media. Dilute plasmid DNA (2 µg) and 6 µL PEI (1 mg/mL) into 100 µL OPTI medium separately. After incubation for 3 min, mix the PEI solution into the DNA solution and incubate at room temperature for 15-20 min. Add the transfection mixture slowly to the cells (200 µL per well) and incubate for 4 h. After then, replace with media with complete DMEM and incubate cells for 24 h-48 h based on the experimental requirements.

**Quantitative Real-Time PCR (RT-PCR)**

Total RNA was extracted using RNA-easy Isolation Reagent (Vazyme), followed by cDNA preparation by the ReverAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer’s instruction. RT-PCR reaction was performed with SYBR Master Mix (Vazyme). Relative mRNA expression for each target gene was calculated by the 2−ΔΔCt method using β-actin as an internal control. Sequences of RT-PCR primers are listed in table S3.

**Inhibitor or activator assay**

Cells were pretreated with different doses of inhibitors or activators dissolved in H2O or 10% DMSO for 2 h, in which stattic was STAT3 inhibitor, AUY922 and 17-AAG were Hsp90 inhibitor, Bazedoxifene was gp130 inhibitor, IL6 and Prostaglandin E2 (PGE2) were IL6-STAT3 pathway activators. Infect cells with indicated viruses for different time points to examine either viral genome entry or replication. Total RNA was extracted for RT-PCR or transcriptomic sequencing analysis (Novegene).

**Transcriptomic analysis**

Transcriptomic sequencing analysis was performed by Novegene company. Briefly, Total Total RNA were extracted using RNA-easy Isolation Reagent (Vazyme), followed by the RNA integrity assessment using the RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). Qualified RNA was subjected to library preparation for transcriptomic sequencing. The clustering of the index-coded samples was performed on a cBot Cluster Generation System (Illumia). After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed using the DESeq2 R package (1.20.0), *P*-value <=0.05. KEGG enrichment analysis of differentially expressed genes was implemented by the cluster Profiler R package, in which gene length bias was corrected, *P*-value <=0.05.

**Protein expression and purification**

Transform pCold-TF-EGFP-VP7 or pCold-TF-EGFP-VP5 plasmid into *E. coli* BL21(ED3). Recombinant His-VP5 or His-VP7 expression was induced by 0.1 mM IPTG at 16 °C overnight. Bacteria were harvested, lysed, and sonicated with lysis buffer (contain 10 mM imidazole and protease inhibitor, pH 8.0). Supernatant was incubated with Ni-beads (Beyotime) for 2 h at 4 °C. Precipitated beads were washed 10-15 times with wash buffer (contain 40 mM imidazole, pH 8.0) and resolved by SDS-PAGE analysis. Samples were then subjected to perform pulldown, immunoblotting or mass spectrometry analysis**.**

**Western blot and Far-western blot**

Cells were harvested and lysed with RIPA lysis buffer (Beyotime) supplemented with protease inhibitor cocktail, Whole cell lysates were sonicated and centrifuged at 12000 rpm at 4 °C for 30 min. Supernatant was resolved with 5×SDS loading buffer by boiling at 98 °C for 10 min. Samples were then separated by SDS-PAGE and followed by transferring to PVDF membrane. Transfer efficiency was checked by Ponceau S staining before probing primary antibody. After that, the membranes were blocked with 4% skimmed milk in TBST for 1 h at room temperature. All immunoblotting analyses were performed using indicated primary antibodies (1:1000-1:2000 dilution in TBST) for 2 h at room temperature and HRP-conjugated secondary antibody (1:2000-1:4000 dilution in TBST) for 1 h at room temperature. Proteins were visualized using ECL detection kit (Beyotime). For far-western blot, cells were prepared as western blot procedure described. After the PVDF membranes were blocked with 4% skimmed milk, incubate the membranes with the purified recombinant protein His-VP7 overnight at 4 °C. The membranes were then subjected to probe with primary anti-His antibody and HRP-conjugated secondary antibody, followed by signal visualization by ECL detection kit.

**CO-IP and His pulldown assays**

Cells were harvested and lysed with NP40 buffer (50 mM Tris-HCl pH 8.5, 150 mM NaCl, 1% NP40) supplemented with protease inhibitor cocktail. WCLs were sonicated for 20 min in ice and centrifugated at 12000 rpm at 4 °C for 30 min. Supernatant was precleared with protein A/G agarose beads at 4 °C for 1 h, and then incubated with corresponding antibody at 4 °C overnight. Agarose beads were washed with lysis buffer and eluted with 1x SDS loading buffer by boiling at 98 °C for 10 min. Samples were then subjected to SDS-PAGE and immunoblotting analysis.

For His pulldown assay, CIK cells were lysed in NP40 buffer supplemented with protease inhibitor cocktail. Cell lysates were centrifugate and precleared with Ni-beads. Precleared cell lysates were incubated with purified recombinant protein His-VP7 or His-VP5. After incubation at 4 °C for 4-6 h, the resin was washed three times with NP40 buffer and then resolved by SDS-PAGE based immunoblotting or mass spectrometry analysis.

**Immunofluorescence microscopy and live cell imaging**

Cells were processed as previously described with some modifications (Li et al., 2019). Briefly, cells expressing fluorescent fusion GFP or RFP were fixed with 4% paraformaldehyde and stained with 4, 6-diamidino-2-phenylindole (DAPI). After that, cells were directly analyzed by immunofluorescence microscope (Nikon). For live cell imaging, CIK cells were seeded on a chambered cover glass at a density of around 50% confluency. Cells were then transfected with corresponding plasmids and videos were recorded using Nikon confocal laser microscope system.

**Subcellular fractionation**

CIK cells (2 x 106) were harvested and washed with 3 mL of cell wash solution. Cell pellet was suspended with 0.75 mL permeabilization buffer to incubate 10 min at 4 °C with constant mixing. Centrifuge permeabilized cells for 15 min at 16,000 × *g* to get the supernatant containing cytosolic protein fractions. 0.5 mL of solubilization buffer was then added to the pellet and incubated at 4 °C for 30 min with constant mixing. Membrane and membrane-associated fractions were obtained by centrifuging the solubilization solution at 16,000 × *g* for 15 min at 4 °C.

**Lentivirus preparation**

Lentiviruses were prepared as previously described (Li et al., 2019). Briefly, HEK293T cells were transfected with packaging plasmids and the lentiviral expression vector. At 48 h post transfection, supernatant was harvested and filtered. Target cells were infected with the supernatant in the presence of polybrene (8 µg/ml) with centrifugation if necessary.

**STAT3 mutant zebrafish generation**

STAT3-KO mutant zebrafish was generated by TALEN technology as previously described (Xiong et al., 2017). Briefly, TALEN right and left arms for STAT3 were designed by TALE-NT program and synthesized using TALEN kit according to the manufacture’s protocol. The linearized TALEN plasmids were transcribed into mRNAs using T7 mMessage mMachine kit. The synthesized mRNAs were purified with lithium chloride precipitation and injected at a dose of 300 pg into the zebrafish embryo at one-cell stage. Genotyping PCR was conducted to determine the mutant zebrafish. The mutated STAT3 codes for a truncated protein containing 165 aa, 46 aa of which at the N-terminal is identical to wildtype STAT3.

**Survival assay**

The experimental koi were kept for a week at 10 °C and were divided into two groups: the koi fish were injected KHV at 20 °C (n=30) and 10 °C (n=30). the control group were injected with PBS at 20 °C (n=30) and 10 °C (n=30). The survival rate was calculated by counting the numbers of dead fish every day.

Mass spectrometry (MS) analysis

Recombinant protein His-VP5 or His-VP7 was purified from *E. coli* BL21(ED3) transformed with plasmid pCold-TF-EGFP-VP5 or pCold-TF-EGFP-VP7 by affinity chromatography using Ni-beads. Purified His-VP5 or His-VP7 was then incubated with CIK whole cell lysate. The incubated beads were washed three times with PBS, followed by adding reaction solution containing SDC, TCEP, and CAA for one-step reduction, alkylation, and elution, respectively. After that, the samples were subjected to enzymatic hydrolysis by trypsin and mass spectrometry analysis using Q EXactive Plus liquid mass spectrometry system (Thermo). The samples were separated by a liquid phase UltiMate 3000 RSLCnano system, with a flow rate being 300 nL per minute.

Electron microscopy

Electron microscope observation of CIK cells infected with GCRV was performed as previously described (Liu et al., 2020). In brief, CIK cells with or without GCRV infection were fixed by 2.5% glutaraldehyde overnight at 4°C. Then the cells were post-fixed in 1% osmium tetroxide for 1 h, followed by washing, dehydration, embedding, and sectioning steps. After that, the cells were stained using 2% uranyl acetate and lead citrate. Images were acquired by transmission electron microscopy (Hitachi-7650, Tokyo, Japan).

**Statistical analysis**

Statistical analysis was performed using unpaired two-tailed Student’s t-test. SPSS was used to analyze the significance test between different groups. A *P*-value <0.05 was considered statistically significant. \**P*<0.05, \*\* *P*<0.01, \*\*\**P*<0.001. Data are represented of three independent experiments and shown as the mean ± SD. For heatmap analysis, transcriptomic data matrix normalized by autoscaling was exported into a txt file and analyzed using TBtools software interface with an in-house R script.

|  |  |  |
| --- | --- | --- |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Antibodies |  |  |
| Rabbit polyclonal hsp90 antibody | Abcepta | Cat#:Azb18696c |
| Rabbit monoclonal stat3 antibody | Beyotime | Cat#:AF1492 |
| Rabbit monoclonal p-STAT3 antibody | Beyotime | Cat#:AF1276 |
| Rabbit monoclonal b-actin antibody | Abclonal | Cat#:AC026 |
| Rabbit monoclonal EEA1 antibody | Bioss Antibodies | Cat#:BS-11250R |
| Rabbit anti his-tag mAb | Abclonal | Cat#:AE086 |
| HRP Goat anti-rabbit antibody | Abclonal | Cat#:AS014 |
| Rabbit polyclonal integrin antibody | This study | N/A |
| Virus Strains |  |  |
| GCRV | This study | N/A |
| CyHV-3 | Lab of L. Zeng | N/A |
| SVCV | Lab of L. Zeng | Wang et al., 2016 |
| MHV68 | Lab of X. Liang | Liu et al., 2020 |
| HSV-1 | Lab of J. Zhang | Sun et al., 2020 |
| AcMNPV | Lab of G. Huang | Yu et al., 2021 |
| GSIV | Lab of L. Zeng | Ma et al., 2014 |
| PEDV | Lab of N. Wang | Tan et al., 2020 |
| Chemicals and Recombinant Proteins |  |  |
| High Affinity Ni-NTA Resin | GenScript | Cat#:L00250-25 |
| nProtein A Sepharose 4 Fast Flow  | GE Healthcare | Cat#:17-5280-01 |
| Polyethylenimine Linear (PEI)  | YENSEN | Cat#:40815ES03 |
| Glutaraldehyde 25% aqueous solution | SINOPHARM | Cat#:111-30-8 |
| Recombinant Hsp90 protein | This study | N/A |
| Recombinant VP5 protein | This study | N/A |
| Recombinant VP7 protein | This study | N/A |
| Critical Commercial Reagents |  |  |
| RNA-easy lsolation Reagent | Vazyme | Cat#:R701-01 |
| First Strand cDNA Synthesis Kit | Thermo scientific  | Cat#:K1622 |
| SYBR qPCR Master Mix | Vazyme | Cat#:Q711-02 |
| Mem-PERTM Plus Kit | Thermo scientific | Cat#:VC301678 |
| Trypan Blue Staining Kit | Beyotime | Cat#:C0011 |
| InStab Protease Cocktail | Yeasen | Cat#20123 |
| Imidazole | Solarbio | Cat#I8090 |
| Experimental Models: Cell Lines |  |  |
| CIK | Lab of T. Xiao | Wang et al.,2017 |
| EPC | Lab of L. Zeng | Meng et al., 2013 |
| CCB | Lab of L. Zeng | N/A |
| GSM | Lab of L. Zeng | Ma et al., 2018 |
| 293T | This study | N/A |
| PK-15 | Lab of N. Wang | Zhang et al., 2019 |
| NHI 3T3 | Lab of X. Liang | Liang et al., 2006 |
| MEF | Lab of X. Liang | Liang et al., 2006 |
| SF9 | Lab of G. Huang | Chen et al., 2017 |
| Experimental Models: Organisms |  |  |
| Zebra fish: STAT3-KO | This study | N/A |
| Zebra fish: WT | This study | N/A |
| Recombinant DNA |  |  |
| pSDred-N1-VP7 | This study | N/A |
| pEGFP-N1-Hsp90 | This study | N/A |
| pCold-TF-GFP-VP7 | This study | N/A |
| pCold-TF-GFP-VP5 | This study | N/A |
| Inhibitor or Activator |  |  |
| Stattic | MCE | Cat#:HY13818 |
| Sodium valproate | AbMole | Cat#:M1876 |
| PGE2 | AbMole | Cat#:M5929 |
| IL6 | Beyotime | Cat#:P5138 |
| NH4Cl | Sinopharm | Cas#:12125-02-9 |
| 17-AAG | MCE | Cat#:HY10211 |
| AUY922 | AbMole | Cat#:M1762 |
| Bazedoxifene | MCE | Cat#:HYA0036 |
| Nystatin | AbMole | Cat#:M3290 |

Table S3

|  |  |
| --- | --- |
| q-PCR primer | Sequence |
| gc-HSP90-qF | CTTTGAGACGGCCACACTGC |
| gc-HSP90-qR | CCTCCTCAGCCTCTGCTTCC |
| gc-IL6-qF | AGAAACTTCAGCACAGAAAGG |
| gc-IL6-qR | ACCTGGAGCTGGCTGTTAAAG |
| gc-βactin-qF | AGCCATCCTTCTTGGGTATG |
| gc-βactin-qR | GGTGGGGCGATGATCTTGAT |
| gc-TNF-α-qF | CATCCATTTAACAGGTGCATAC |
| gc-TNF-α-qR | GCAGCAGATGTGGAAAGAGAC |
| gc-IL-1β-qF | GATTCGAAAGTTCGATTCAATCT |
| gc-IL-1β-qR | TTCAGTGACCTCCTTCAAGAC |
| gc-LamR-qF | CGGGATCCATGTCCGGAGGTCTGGAT |
| gc-LamR-qR | CCGCTCGAGAGACCAGTCGGCAGCGGT |
| gc-SRB1-qF | AGTTTCAGGCATCACAGAAACAGGA |
| gc-SRB1-qR | GCTGAGGTATAATATCACGGCTCCC |
| gc-JAMA-qF | TGTGGCTTTGCTGGCAGTAG |
| gc-JAMA-qR | TGGTTGCTTTCTGCTATTTTT |
| gc-gp130-qF | GGGTCAATGCTCTGCTTGCG |
| gc-gp130-qR | CACCTGGTCCTCCACCTTGG |
| gc-Inte4-qF | AGCGCATAATGTTCTGTGCTTCA |
| gcInte4-qR | CCAACCTCCAAGTCACCAAAGTG |
| gc-IL6R-qF | TCTTGGCAGCCACTTGGTCA |
| gc-IL6R-qR | CAGCCACGCAGTAGCATGTC |
| gc-HSF-qF | AGCTCGGAATCGCCTGTGAA |
| gc-HSF-qR | AGGACACACCTCCTCCACCT |
| gc-E-cadherin-qF | CCAGTGGCAAGAAGCACACG |
| gc-E-cadherin-qR | TCAAGAACACGGAACGGAGGA |
| gc-Fibronectin-1β-qF | TGCGCTACGGACCAAGAGAG |
| gc-Fibronectin-1β-qR | TGGGAGGTCAAGCACACGTT |
| gc-Fibronectin-1α-qF | TGACCAGGACACACGCACTT |
| gc-Fibronectin-1α-qR | ACAGGATCCCTCCACAGGGT |
| gc-RAB5A-qF | CAGGAGCGCTACCACAGTCT |
| gc-RAB5A-qR | CTCTTGCTGGCAAGGTCTGC |
| gc-Myoal-qF | CCATGGTGGAGCGCAGAAAC |
| gc-Myoal-qR | ATGCACGAGGTCAGCATCCA |
| VP7-qF | CCATGACACTCACGCACACG |
| VP7-qR | GGCAAGCGAAGGTCAGGTTG |
| CyHV-3 ORF1L-qF | TGCGCTCAGCAAGAAGTACCA |
| CyHV-3 ORF1L-qR | CGCCGCACAGTAGTTGAAAAA |
| CyHV-3 ORF4L-qF | GTCGTGCAGTCGTGGACCT |
| CyHV-3 ORF4L-qR | CCAGAGCATAGGCATCAGCC |
| Carp-IL6-qF | CAGATAGCGGACGGAGGGGC |
| Carp-IL6-qR | GCGGGTCTCTTCGTGTCTT |
| GSIV-qF | GAGCAGCACAGTCAGGGACA |
| GSIV-qF | ACGGAAGGGTGTGTGACGTT |
| GSM-βactin-qF | CCCAAAAGCCAACCGAGAAA |
| GSM-βactin-qR | GACACCATCACCAGAGTCCA |
| AcMNPV ACp35-qF | GGTCGACGAACGCAACGACTACT |
| AcMNPV ACp35-qR | TAATTTCGTGAGCAAACGGCACA |
| sf9-βactin-qF | AGCGTTTGACGCTACTGGGT |
| sf9-βactin-qR | CTCCCTGTTCTCAGGCGTGT |
| pig-βactin qF | AGCGCAAGTACTCCGTGTG |
| pig-βactin qR | CGGACTCATCGTACTCCTGCTT |
| PEDV-qF: | GCTAGTGGCGTTCATGG |
| PEDV-qR: | TGTAAATAAAGCTGGTAACCAC |
| MHV68 ORF50-qF:  | CAGTTGGCCTGGGCATATG |
| MHV68 ORF50-qR: | GTATGTCGTCAGTGGGCAACTC |
| MHV68 ORF59-qF:  | TGACTGGCAGGTTTTTGTATGC |
| MHV68 ORF59-qR:  | GATGATCGTGAGGCCAATGG  |
| mIL-6-qF: | TCCAGTTGCCTTCTTGGGAC |
| mIL-6-qR: | GTACTCCAGAAGACCAGAGG |
| mβ-actin-qF | AAATCGTGCGTGACATCAAAG |
| mβ-actin-qR | AAGAAGGAAGGCTGGAAAAGAG |
| HSV-1 vp23-qF | CAACTCGGTCCCGTAAACGC |
| HSV-1 vp23-qR | CGCGTGACACTCTGCATCTG |
| HSV-1 vp26-qF | GCCGTCCCGCAATTTCACC |
| HSV-1 vp26-qR | GGTGCGGGTGGTTGTTATCC |
| hβ-actin-qF | GGCACTCTTCCAGCCTTCCT |
| hβ-actin-qR | GCCAGGGCAGTGATCTCCTT |
| hIL6-qF | GAAGCTGCAGGCACAGAACC |
| hIL6-qR | TGTGCCCAGTGGACAGGTTT |
| HSV-1 vp23-qF | CAACTCGGTCCCGTAAACGC |
| HSV-1 vp23-qR | CGCGTGACACTCTGCATCTG |
| HSV-1 vp26-qF | GCCGTCCCGCAATTTCACC |
| HSV-1 vp26-qR | GGTGCGGGTGGTTGTTATCC |
| SARS-COV-2 S-qF | TGCACAGAAGTCCCTGTTGCT |
| SARS-COV-2 S-qR | GCCCGCCGAGGAGAATTAGT |

FIGURE LEGENDS

**Figure S1.** **Temperature dependency of GCRV infection**

(**A**) Grass carp hemorrhagic disease survey from 46 fisheries distributed in Yiyang and Changsha city in Hunan province were investigated.

(**B**) Hemorrhagic symptoms were observed in gills, fins, skins, and muscles from figure 1B.

(**C**) The relative GCRV genome replication from infected GCO cells under different temperatures was analyzed by RT-PCR.

(**D**) Schematic of experimental design showing temperature stress affects GCRV replication in CIK cells.

(**E**) The relative GCRV genome replication from 28 °C of infection switch to 18 °C of infection was analyzed by RT-PCR. \**p*<0.05 and \*\**p*<0.01.

**Figure S2. IL6-STAT3 signaling pathway mediates temperature dependent GCRV infection**

(**A-B**) Gills (**A**) and intestines (**B**) of grass carp under temperature stress from 18 °C to 28 °C were prepared to analyze the relative expression of proinflammatory genes.

(**C**) CIK cells infected with GCRV under different temperature were prepared to quantify the relative expression of gp130, IL6R and integrin-α4.

(**D-E**) Gills of grass carp infected with GCRV at indicated timepoints were prepared to quantify the relative viral genome replication and expression of IL6 by RT-PCR.

**Figure S3. IL6-STAT3-HSP90 signaling axis mediates temperature dependency of GCRV infection**

(**A-B**) 293T cells were transfected with different dose of STAT3-C and harvested to quantify the relative expression of IL6 (**A**) and HSP90 (**B**).

(**C**) CIK cells transfected with pEGFP-Hsp90 were prepared to analyze the subcellular localization of HSP90 by fluorescence microscopic analysis. Scale bars denotes 20 μm.

(**D**) CIK cells under temperature stress were prepared for endogenous co-immunoprecipitation analysis with STAT3 antibody to examine the interaction between HSP90 and STAT3.

(**E**) Transcriptomic data from CIK cells under temperature stress from 18 °C to 28 °C were prepared to analyze the differentially regulated heat shock protein related genes by heat map analysis.

(**F-G**) Gills of grass carp (**F**) or CIK cells (**G**) under temperature stress from 18 °C to 28 °C were prepared to analyze the relative expession of HSP90 by RT-PCR.

(**H**) CIK cells infected with GCRV at indicated timepoints were prepared to analyze the relative expression of HSP90.

(**I**) CIK/GCO cells under temperature stress or GCRV infection were prepared to analyze the expression of HSP90 by western blotting analysis.

**Figure S4.** **IL6-STAT3-HSP90 signaling axis regulates GCRV entry**

(**A**) IL6-STAT3-HSP90 signaling axis activators or inhibitors used in this study.

(**B**) CIK cells treated with different dose of PGE2 were prepared to analyze the relative expression of IL6 by RT-PCR.

(**C**) GCO cells transfected with different dose of HSP90 plasmids were infected with GCRV for 1 h and harvested to quantify the relative viral genome entry level by RT-PCR.

(**D-E**) 293T cells transfected with different dose of HSP90 plasmids were infected with GCRV for 1 h and harvested to quantify the relative viral genome entry level (**D**) and expression of HSP90 (**E**) by RT-PCR.

(**F**) CIK cells pre-treated with different dose of AUY922 were infected with GCRV for 1 h and harvested to quantify the relative viral genome entry level by RT-PCR.

(**G**) GCRV pre-incubated with different dose of HSP90 were employed to infect CIK cells for 1 h, after then, cells were prepared to analyze the relative viral genome entry level by RT-PCR.

(**H**) Transcriptomic data from CIK cells treated with HSP90 inhibitor 17-AAG were prepared to perform a GO enrichment analysis.

**Figure S5.** **HSP90 interacts with VP7 and STAT3 on cellular membrane to promote GCRV entry**

(**A**) Mass spectrometry analysis of pulldown complex by purified VP5-HIS-EGFP identified HSP90 as a target of VP5.

(**B**) Pulldown analysis was conducted to confirmed the interaction between VP5/VP7 and HSP90 in CIK cells.

(**C**) Pulldown analysis were conducted to determine the interaction between STAT3 and VP7 in CIK cells.

(**D**) CIK cells treated with 17-AAG were prepared to examine the interaction between VP7 and HSP90 by pulldown analysis.

(**E**) GCO, CIK or 293T cells stably expressing tagged VP7 and HSP90 were prepared to analyze their colocalization by immunofluorescence analysis. Scale bars: 20 μm.

(**F**) CIK cells pre-treated with different dose of VP7 were infected with GCRV for 1 h and prepared to analyze the relative viral genome entry by RT-PCR analysis.

(**G**) CIK cells pre-treated with VP7 in the presence or absence of AUY922 were infected with GCRV for 1 h and prepared to quantify relative viral genome entry level by RT-PCR. (**H**) CIK cells pre-treated with VP7 were infected with GCRV for 1 h and prepared to quantify the relative expression of many plasma membrane related genes by RT-PCR.

**Figure S6.** **IL6-STAT3-HSP90 signaling axis mediates viral entry in ectotherms**

(**A**) Similarity analysis of amino acids from IL6-STAT3-HSP90 signaling axis related proteins were conducted by multiple sequence alignment program.

(**B**) Predicted three dimentional structures of IL6-STAT3-HSP90 signaling axis related protein were performed with I-TASSER program and visualized with PyMOL program.

(**C**) The relative expression of ORF4L from lytic infection of KHV was analyzed by RT-PCR.

(**D-E**) GSM cells pre-treated with IL6-STAT3-HSP90 signaling axis inhibitors were infected with GSIV for 1 h and prepared to quantify the relative viral genome entry level by RT-PCR.

(**F**) GSM cells pre-treated with IL6-STAT3-HSP90 signaling axis related activators or inhibitors were infected with GSIV for 1 h and prepared to quantify the relative viral genome entry level by RT-PCR.

**Figure S7.** **IL6-STAT3-HSP90 signaling axis mediates viral entry in endotherms**

(**A**) MEF cells under lytic infection of MHV68 were prepared to quantify the relative expression of ORF59 by RT-PCR.

(**B**) 293T cells under lytic infection of HSV1 were prepared to quantify the relative expression of VP26 by RT-PCR.

(**C-D**) 293T cells pre-treated with different dose of stattic, AUY922 (**C**), or PGE2 (**D**) were infected with HSV1 for 1 h and prepared to quantify the relative viral genome entry level by RT-PCR.

(**E**) Schematic diagram of experimental design showing the preparation of SARS-COV-2 pseudovirus by lentiviral vector based transfection.

(**F**) 293T-ACE2 cells pre-treated with different dose of AUY922 or bazedoxifene were infected with SARS-COV-2 pseudovirus and prepared to quantify the relative viral genome entry level by RT-PCR.

(**G**) PK cells pre-treated with different dose of IL6, PGE2, or 17-AAG were infected with PEDV for 1 h and prepared to quantify the relative viral genome entry level by RT-PCR. (**H**) Cells treated with IL6-STAT3-HSP90 signaling axis related drugs were analyzed by trypan blue staining and cell viability was determined.

**Legends for supporting videos**

Supplementary video 1: CIK cells transiently transfected with pSDred-N1 vector plasmid were recorded using Nikon confocal laser microscope system.

Supplementary video 2: CIK cells transiently transfected with pSDred-N1-VP7 plasmid were recorded using Nikon confocal laser microscope system.