WITHDRAWN: Temperature-dependent IL6-STAT3-HSP90 signaling mediates viral entry

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Abstract

IL6-STAT3 signaling is a canonical pro-inflammatory pathway that is evolutionarily conserved and plays important roles in both temperature stress and virus infection. However, our understanding of the molecular mechanisms underpinning the temperature-dependent pathogenesis of viral infection in ectotherms remains rudimentary. Here we report that Grass carp reovirus (GCRV) hijacks the temperature-dependent IL6-STAT3-HSP90 signaling axis to promote viral entry. Exploring the temperature-dependent phenotype of GCRV infection, we discovered that the activation of the IL6-STAT3 signaling facilitated viral entry by increasing the expression of heat shock proteins (HSPs). Further biochemical, transcriptomic, and microscopic analyses revealed that major capsid protein VP7 of GCRV interacted with HSP90 and relevant cytoskeleton proteins to enable viral entry. Accordingly, exogenous expression of either IL6, HSP90, or VP7 in cells engendered an increased viral entry in a dose dependent manner. Interestingly, other viruses infecting invertebrate insects, ectothermic vertebrates (e.g., fish, amphibians), and endothermic mammals (e.g., swine, mouse, human) have evolved similar mechanism to promote their infection. Altogether, our work delineates a pivotal role of the IL6-STAT3-HSP90 signaling axis in the temperature-dependency of viral entry, pinpointing the feasibility of developing targeted therapies for viral diseases in the future.

Introduction

Inflammation is an evolutionarily conserved process characterized by the activation of immune and non-immune cells that protect the host by eliminating pathogen infection and promoting tissue repair and recovery (Netea et al., 2017). However, aberrant inflammation, most commonly in the form of chronic inflammation, can, in turn, engender many diseases that collectively represent the leading causes of disability and mortality worldwide, such as cardiovascular disease, diabetes mellitus, chronic kidney disease, autoimmune diseases, cancer, and neurodegenerative diseases (Furman et al., 2019). Studies have shown that canonical biomarkers of acute inflammation, such as IL6, C-reactive protein (CRP), IL1-β, TNF-α, are closely associated with the fore-mentioned diseases. Accordingly, plenty of preclinical and clinical studies targeting these biomarkers are now being pursued to help unravel the mechanisms and translate them into effective preventives and strategies for human health (Furman et al., 2019).

Virus-host interaction is a continuous coevolutionary process involving host develop strategies to limit virus infection and viruses exploit counter-adaptation mechanism to survive (Kajan et al., 2020). As viruses are obligate intracellular parasites, they frequently rely on and hijack host machineries, including the host immune system to facilitate their viral life cycle. Emerging evidence shows that many classical immune-surveillance networks, which are supposed to protect the host from viral infections, are being hijacked by viruses to ensure their own survival, e.g., NF-kappa B signaling, IL6/STAT3 signaling, autophagy, cytokines, cellular membranes, heat shock proteins (HSPs), and cytoskeletons (Kajan et al., 2020; Kikkert, 2020; Yang and Shu, 2020). Among them, IL6/STAT3 signaling, an intrinsic antiviral and pro-inflammatory pathway, has been suggested to contribute several viral disease progression (Kuchipudi, 2015), while numerous investigations through blockage or inhibition of key

components of IL6/STAT3 signaling have proven the importance of IL6/STAT3 signaling in virus infection (Gubernatorova et al., 2020; Kuchipudi, 2015; Sen et al., 2012). Nevertheless, the exact molecular mechanism regarding how viral life cycle is governed by IL6/STAT3 signaling remains largely unexplored.

Temperature is one of the key elements regulating all kinds of biological activities on earth. Compared to endotherms, viral diseases in ectotherms show typical seasonality and temperature dependency, which caused enormous losses worldwide in aquaculture industry (Pereiro et al., 2021; Reverter et al., 2020). Interestingly, IL6/STAT3 signaling is a unique and highly pleiotropic pathway that is evolutionarily conserved and is implicated in temperature-induced stress responses (Gubernatorova et al., 2020; Kuchipudi, 2015; Rummel et al., 2006). On the other hand, viral entry is the first step for establishing an infection and launching a viral disease. To date, there is no report of the interplay among temperature, IL6/STAT3 signaling and viral entry.

Grass carp reovirus (GCRV) is the causative agent of a lethal, highly contagious and notifiable disease in grass and black carp (Zhang et al., 2010), two important freshwater products in China. In this study, we employed the infection of grass carp by GCRV as a homologous virus-host model to dissect the intricate molecular mechanism behind seasonality and temperature dependency of virus infection in aquatic ectotherms. We discovered that GCRV hijacked the temperature dependent IL6-STAT3-HSP90 signaling axis to enable viral entry. Mechanistically, major capsid protein VP7 of GCRV interacted with HSP90 and relevant cytoskeleton proteins to promote viral entry. Exogenous expression of either HSP90 or VP7 in cells rendered an increased viral entry therefrom. Importantly, we found that such mechanism may be conserved in many other viruses, including DNA or RNA viruses from invertebrate insects, ectothermic vertebrates (e.g., fish, amphibians), and endothermic mammals (e.g., swine, mouse, and human). Our work delineated a pivotal role of IL6-STAT3-HSP90 signaling axis in the temperature dependency of virus entry.

Results

Temperature-dependency phenotype of GCRV infection

Grass carp hemorrhagic disease (GCHD) in grass carp is caused by an aquatic reovirus (GCRV) infection. Compared with endotherms, viral diseases in lower ectotherms are more susceptible to temperature fluctuation. This hypothesis is driven by the fact that summer to autumn (May to September) in south of China is a peak period of hemorrhagic disease incidence when mean temperature and diurnal variation tend to be high (Fig. 1a and Extended Data Fig. 1a). An outbreak of hemorrhagic disease of grass carp under natural condition in mid-September was recently documented. The water temperature increased by 3 degrees from 11^{th} to 14^{th}, when GCHD began and the number of deaths surged to 500 by 14^{th} Sep. From 14^{th} to 18^{th}, the temperature as well as the number of deaths declined sharply due to rainy weather (Fig. 1b). The mortality curve coincided well with the temperature variation curve, with the number of deaths peaked at around 28 °C and recovered from the disease outbreak at around 18 °C (Fig. 1b),

suggesting that temperature stress may play an important role during the outbreak and alleviation of GCRV disease. The sick fish showed typical inflammatory and hemorrhagic symptoms on the gill cover (a), fin (b), skin (c), and intestine (d) (Extended Data Fig. 1b). RT-PCR analysis showed that compared with asymptomatic fish at 18 °C, the viral genome level is much higher in tissues from symptomatic fish at 28 °C (Fig. 1c). To dissect the molecular mechanism underlining the temperature dependency of GCRV infection, we performed the *de novo* GCRV infection at 18 °C and 28 °C at cellular level to represent resistant infection and permissive infection, respectively. Electron microscopy analysis of GCRV infected grass carp (*Ctenopharyngodon idellus*) kidney (CIK) cells showed that viral infection at 28 °C displayed more typical cytopathogenic effect (CPE) and more virion particle array within cells (Fig. 1d). Consistent with the microscopic data, RT-PCR analysis showed that the viral proliferation rate at 28 °C was almost two orders of magnitude faster than 18 °C in CIK cells (Fig. 1e). Similar results were obtained from grass carp ovary (GCO) cells infected at different temperature (Extended Data Fig. 1C). To determine the impact of temperature difference or temperature stress on viral proliferation, we conducted a temperature-switch experiment. We infected CIK cells at 18 °C or 28 °C for 8 h, then switched the temperature to 28 °C or 18 °C, respectively (Extended Data Fig. 1D). RT-PCR analysis showed that virus proliferation surged 10-fold by 18 °C to 28 °C switch (Fig. 1f), while 28 °C to 18 °C switch significantly slowed the viral genome replication by a factor of 5 to 32 (Extended Data Fig. 1E), confirming the vital role of temperature on viral infection and proliferation. Collectively, these results support the conclusion that the epidemiology and pathology of GCRV infection shows a temperature-dependent phenotype.

Temperature dependency of GCRV infection counts on the activation of IL6/STAT3 signaling

To delineate the mechanism of temperature dependency of GCRV infection, we hypothesized that the temperature related immune responses may be exploited by virus to promote its infection efficiency and propagation later on. Based on a previous transcriptomic data of GCRV infected grass carp or CIK cells (He et al., 2017), as shown by the volcano plot (Fig. 2a) and heat map (Fig. 2b) analyses, GCRV infection significantly activated proinflammatory pathways, including NF-kappa B and IL6/STAT3 (Fig. 2a, b). IL6, IL1β, and TNFα are canonical cytokines that serve as pivotal mediators of inflammatory responses. In addition, circulating IL6 from peripheral sites could act as a messenger that transmits inflammatory information to the central nervous system (CNS) to mediate temperature related responses through IL6-STAT3 signaling (Evans et al., 2015; Rakus et al., 2017; Rummel et al., 2006). We thus hypothesized that proinflammatory cytokines, IL6 particularly, are engaged in the temperature dependency of GCRV infection. Indeed, RT-PCR analysis of the gill samples from natural outbreak of GCHD in fish ponds (Fig. 1b, c) showed that higher expression of IL6, IL1β and TNF-α positively correlated with faster GCRV proliferation at 28°C (Fig. 2c and Fig. 1c). To determine the role of temperature on viral infection and IL6/STAT3 signaling, we firstly evaluated the effect of temperature stress on the immune responses of grass carp tissues or CIK cells. In the absence of viral infection, a temperature switch from 18 °C to 28 °C for CIK cells, or for grass carp tissues in the fish tank, significantly induced the expression of IL6, IL1β and TNF-α (Fig. 2d and Extended Data Fig. 2a, b). The elevation of IL6 was most evident, as it reached the peak rapidly in CIK cells (Fig. 2d), gills, and intestines of grass carp (Extended Data Fig. 2a, b). Consistently, GCRV infection on CIK cells induced the expression of pyrogenic cytokines (IL6, IL1β)

and plasma membrane receptors (gp130, IL6R, integrin-α4) in a temperature-dependent manner (Fig. 2e and Extended Data Fig. 2c). Notably, asymptomatic infection at 18 °C barely induced the expression of IL6 and IL1β, while higher temperature of 28 °C infection significantly induced their expression (Fig. 2e), suggesting that GCRV exploits IL6 signaling to facilitate its infection at 28 °C. In addition, RT-PCR analysis of spleen and gill samples from grass carp artificially infected by GCRV further confirmed that the replication of GCRV genome paralleled with induced expression of IL6 (Fig. 2f, g and Extended Data Fig. 2d, e), signifying our hypothesis that the expression of IL6 facilitated GCRV infection. As STAT3 is a transcription factor downstream of IL6 signaling activation, we examined whether IL6/STAT3 signaling axis mediates temperature-dependency of GCRV infection. By western blotting analysis with STAT3 activation specific antibody (STAT3-P, Y705), we further confirmed that both temperature stress and GCRV infection activated STAT3 (Fig. 2h). Conversely, STAT3 inhibition by stattic greatly reduced the replication of GCRV by 86% (Fig. 2i), which also impaired the GCRV genome replication and IL6 expression a dose dependent manner (Fig. 2j, k), suggesting that IL6/STAT3 activation is essential for GCRV proliferation. Together, these data indicate that IL6/STAT3 signaling activation is crucial to temperature-dependency of GCRV infection.

IL6-STAT3-HSP90 signaling axis mediates temperature dependency of GCRV infection

It has been previously reported that STAT3 is a client protein of HSP90, through a direct or indirect manner. HSP90, together with IL6/STAT3 signaling, is essential for the progression of various diseases, infections, and cancers (Kolosenko et al., 2014; Prinsloo et al., 2012; Whitesell and Lindquist, 2005; Wyler et al., 2021). Thus, we postulated that IL6/STAT3 signaling might regulate HSP90 expression to engage viral infection. To probe the role of HSP90, we first generated STAT3 deficient zebrafish mutant by TALEN-based genome editing technology (Fig. 3a-c). STAT3-KO mutant codes for a truncated protein containing only 46 aa of the N-terminus of STAT3-WT. Transcriptomic analysis showed that compared with STAT3-WT, the expression of heat shock protein related genes in STAT3-KO mutant was greatly reduced. Importantly, STAT3-KO mutant almost abolished the expression of HSP90 (Fig. 3d), suggesting that HSP90 is a downstream target gene of STAT3 signaling. Consistently, STAT3 inhibition by stattic treatment in CIK cells dampened the expression of HSP90 in a dose dependent manner (Fig. 3e). Conversely, overexpression of STAT3-C (A661C, D663C), an constitutively active mutant of STAT3 (Takahashi and Yamanaka, 2006), upregulated the transcription of IL6 and HSP90 (Extended Data Fig. 3a, b), indicating that HSP90 is indeed a downstream gene of STAT3 signaling. In addition, immunofluorescence analysis showed that HSP90 mainly located on the plasma membrane in resting cells (Extended Data Fig. 3c), while temperature stress from 18 °C to 28 °C in CIK cells induced the activation of STAT3 and its trafficking from cytoplasm to cellular membrane fraction where HSP90 located, suggesting that temperature stress induced the association between STAT3 and HSP90 (Fig. 3f). Similarly, endogenous co-immunoprecipitation analysis from CIK cells infected with GCRV confirmed that STAT3 bound to HSP90 (Extended Data Fig. 3d). To probe the role of HSP90 in the temperature-dependency of GCRV infection, we conducted a transcriptomic analysis of CIK cells under heat stress treatment from 18 °C to 28 °C. The volcano plot and heat map analyses showed that HSP90, together with other HSPs were significantly induced by heat stress (Fig. 3g, Extended Data Fig. 3e). RT-PCR

analysis of intestines, gills and CIK cells from heat stress further confirmed that the transcription of HSP90 was induced by the treatment (Fig. 3h, Extended Data Fig. 3f, g). In addition, RT-PCR analysis of CIK cells further showed that GCRV infection induced the expression of HSP90 in a temperature dependent manner, from which asymptomatic infection at 18 °C barely induced the expression of HSP90, while higher temperature of 28 °C infection significantly induced the expression of HSP90 (Fig. 3i, Extended Data Fig. 3h), suggesting that GCRV may exploit the expression HSP90 to facilitate viral infection at 28 °C. Western blotting analysis further validated the elevated protein level of HSP90 under heat stress from 18 °C to 28 °C or GCRV infection at 28 °C (Extended Data Fig. 3i). Finally, CIK cells treated with 17-AAG, a HSP90 specific inhibitor, greatly blocked the proliferation of GCRV in a dose dependent manner (Fig. 3j). Collectively, these data indicates that HSP90, a downstream gene of STAT3 signaling, associates with STAT3 and plays vital roles in temperature dependency of GCRV infection.

IL6-STAT3-HSP90 signaling axis regulates GCRV entry

To determine which step of viral life cycle is governed by IL6-STAT3-HSP90 signaling, we first examined the viral genome copy number in CIK and GCO cells within 1 h of infection, which is supposed to be the initial viral DNA upon viral entry (Byk et al., 2016). RT-PCR analysis showed that compared with 18 °C, infection at 28 °C in CIK or GCO cells increased the viral genome number by a factor of 5 to 40 (Fig. 4a), suggesting that temperature plays an important role in the infectivity of GCRV. Given that HSP90 was reported to function as co-receptor of many viruses (Srisutthisamphan et al., 2018; Zhang et al., 2020), we hypothesized that IL6-STAT3-HSP90 signaling mediates GCRV initial infection during the viral entry stage, and was further exploited by the virus for subsequent viral spreading. To test this, we employed several IL6-STAT3 signaling specific activators or inhibitors to treat cells for 2 h, then infected cells for 1 h with GCRV to quantify viral entry (Extended Data Fig. 4a). RT-PCR analysis showed that treating cells with IL6-STAT3 signaling inhibitors including bazedoxifene (Fig. 4b) and stattic (Fig. 4b-d) diminished the infection efficiency of GCRV by 50% to 70%, while IL6-STAT3 signaling activators, including IL6, prostaglandin E2 (PGE2), or valproic acid (VPA) significantly increased the infection efficiency (Fig. 4e). Both activators and inhibitors had a dose dependent effect on viral infection (Fig. 4c-e). Next, we determined whether HSP90 regulates GCRV entry. We found that overexpression of HSP90 in CIK and GCO cells significantly increased the viral entry in a dose dependent manner (Fig. 4f, Extended Data Fig. 4c), Moreover, overexpression of grass carp HSP90 in 293T cells, which is a non-permissive cell for GCRV, enabled viral entry (Extended Data Fig. 4d-e). On the contrary, HSP90 inhibition by two inhibitors, including AUY922 and 17-AAG, strongly impaired viral entry (Fig. 4g and Extended Data Fig. 4f). Interestingly, when GCRV was pre-incubated with different dose of HSP90, viral entry was greatly blocked (Extended Data Fig. 4g), suggesting that HSP90 may function as a co-receptor on the plasma membrane to mediate viral entry. To further dissect the role of HSP90 in viral infection, we performed a transcriptomic analysis of CIK cells treated with HSP90 specific inhibitor 17-AAG, we found that the transcription of membrane receptors, cell communication, cytoskeleton protein, and ion channel related proteins, which mostly participate in many fundamental biological processes (Fig. 4h). Further KEGG pathway analysis showed that cell adhesion and cell communication related pathways were significantly downregulated when HSP90 was inhibited (Extended Data Fig. 4h), suggesting HSP90 plays important

roles in viral initial infection. Combined these results with primary plasma membrane localization of HSP90 by previous data, it is conceivable that HSP90 locates on the outer membrane of the cells, which may function as a co-receptor or key chaperon, binds to bona fide viral receptors and structure proteins of GCRV to facilitate initial viral infection.

HSP90 interacts with VP7 of GCRV to promote viral entry

It has been previously reported that VP5 and VP7, the major outer capsid proteins of GCRV, are extremely important for establishing infection and have been well investigated as the potential vaccination target against GCRV infection (Zhang et al., 2010). We thus hypothesized that HSP90 may interact with them to facilitate GCRV entry. We first purified double-tagged fusion capsid proteins of GCRV, His-GFP-VP5 and His-GFP-VP7, by Ni-NTA based affinity purification to homogeneity (Fig. 5a-5b). We then incubated these fusion proteins with GCO cells, microscopic analysis found that they bound to the outer membrane of GCO cells (Fig. 5c), indicating they are the immunogens of GCRV to mediate viral entry. To test whether VP5 and VP7 bind to HSP90 on the host cells to facilitate GCRV entry, we incubated tagged VP5 and VP7 with lysates of CIK cells and sent them for mass spectrometric analysis. Both samples from VP5 and VP7 identified HSP90 as their binding targets (Fig. 5d, Extended Data Fig. 5a), as well as cytoskeletal proteins (e.g., tubulin, actin, actinin), chaperone proteins (e.g., HSP70, HSP60), and transport proteins (e.g., clathrin, dynein, kinesin, importin) (Supplementary Table 1-2), suggesting that VP5 and VP7 participated in the initial viral entry and transportation during GCRV infection. To verify the interaction between VP5/VP7 and HSP90, we performed a His pulldown assay using His-VP5/VP7 fusion proteins. The data confirmed that both VP5 and VP7 interacted with HSP90, with a much stronger interaction between VP7 and HSP90 (Extended Data Fig. 5b), suggesting that VP7 plays indispensable roles in HSP90 mediated GCRV infection. Given that HSP90 associated with STAT3 on the cellular membrane during temperature stress or GCRV infection, we examined whether VP7 interacted with HSP90 and STAT3 during GCRV infection. Fractionation coupled with His pulldown analysis showed that, compared with VP5, VP7 was strongly associated with HSP90 and STAT3 in both the membrane and cytosolic fraction, where early endosome marker EEA1 resided (Fig. 5e, Extended Data Fig. 5c), suggesting that STAT3, HSP90, and VP7 may function as a complex facilitating viral entry. To further examined the direct interaction between VP7 and HSP90, we performed a far-western blotting analysis, the results showed that HSP90 on the PVDF membrane could interact directly with VP7 *in vitro* (Fig. 5f), while the interaction was blocked by HSP90 inhibitor treatment (Extended Data Fig. 5d). Furthermore, immunofluorescence analysis showed that VP7 colocalized with HSP90 to form aggregate or punctate signals in GCO, CIK and 293T cells (Fig. S5E). To probe the role of VP7 in GCRV infectivity, we found that pre-treated CIK cells with purified VP7 increased the viral entry of GCRV in a dose dependent of manner (Extended Data Fig. 5f), suggesting that VP7 pre-treatment could get the cells ready for viral entry. Conversely, with endocytosis inhibitors pre-treatment, such as ammonium chloride or nystatin, or pre-treated GCRV particles with VP7 protein, which is supposed to block the effect of VP7 on GCRV infection, nearly abolished the infection of GCRV (Fig. 5g). Also, HSP90 inhibitor AUY922 treatment recapitulated the effect of endocytosis inhibition or VP7 blockage (Extended Data Fig. 5g). To probe the role of VP7 on host cells, we first performed a live cell imaging analysis of CIK cells stably expressing VP7 (Supplementary Video 1-2), we found that compared

with the evenly distributing pattern of vector, VP7 displayed a more clustered pattern, which pulled the adjacent VP7 expressing cells together to form a bigger aggregate (Supplementary Video 1-2), suggesting that VP7 plays important roles mediating viral spreads through cell-to-cell transmission. To further examine the subcellular structural change of host cells caused by VP7, we performed an electron microscopy analysis of CIK cells treated with purified VP7 protein, we found that CIK cells showed a typical fuzzy morphology on the plasma membrane when cells were treated with purified VP7 protein (Fig. 5h), suggesting that the morphologic change on the plasma membrane may enable the landing and wrapping of virion particles during the initial infection stage. RT-PCR analysis further found that VP7 increased the expression of many genes that involved in cell-to-cell communication and viral infection, including fibronectin, myosin, integrin, laminin receptor (LamR), and scavenger receptor class B type 1 (SRB1) (Extended Data Fig. 5h). Collectively, these results supported the conclusion that VP7 interacts with HSP90 and STAT3 on cellular membrane fraction during GCRV infection, and promotes viral entry by inducing cytoskeletal protein rearrangement and expression of plasma membrane receptors, which ultimately get the cell ready for viral entry.

IL6-STAT3-HSP90 signaling axis mediates viral entry in aquatic ectotherms

To determine whether the mechanism of IL6-STAT3-HSP90 axis regulating viral entry applies to other virus-host interactions, including ectotherms and endotherms, we first performed the amino acid alignment of IL6-STAT3-HSP90 axis related proteins across species, including IL6, IL6R, gp130, JAK1, STAT3 and HSP90. We found that the sequence similarity was higher for downstream proteins within this axis (Extended Data Fig. 6a). For example, the similarity score was over 85% in all kinds of vertebrates STAT3 and HSP90 (Extended Data Fig. 6a). For STAT3, the key amino acids that are required for phosphorylation-mediated activation was highly conserved (Fig. 6a). In addition, the predicted three-dimensional structures of IL6-STAT3-HSP90 axis related proteins in grass carp and human showed similar spatial structure (Extended Data Fig. 6b), suggesting that the signaling transduction pathway is conserved during evolution. To explore whether IL6-STAT3-HSP90 axis regulates viral infection in other aquatic ectotherms, we examined the Koi herpesvirus (KHV) and Spring viremia of carp virus (SVCV) in Koi and carp, respectively. The survival rate analysis of KHV infection in Koi showed typical temperature dependency, in which higher temperature of infection greatly increased the mortality of Koi, while lower temperature of infection tended to preserve their survival (Fig. 6b). RT-PCR analysis showed that lytic infection of KHV in common carp brain (CCB) cells (Fig. 6c, Extended Data Fig. 6c) or heat stress of Koi (Fig. 6d) significantly induced the expression of IL6, suggesting that temperature-dependent IL6 expression may participate in the lytic infection of KHV. Furthermore, STAT3 inhibitor stattic or HSP90 inhibitor AUY922 treatment reduced the KHV entry during the early step of infection (Fig. 6e-f). The same held true with AUY922 treatment to SVCV infection (Fig. 6g). Furthermore, we tested whether this mechanism can be extended to vertebrate amphibian and invertebrate ectotherms. We employed aforementioned inhibitors or activators to Chinese giant salamander muscle cells (GSM) or Sf9 insect cells, then infected them with Chinese giant salamander iridovirus (GSIV) (Fig. 6h, Extended Data Fig. 6d-e) or baculovirus AcMNPV (Fig. 6i, Extended Data Fig. 6f), respectively. RT-PCR analysis showed that inhibition of IL6-STAT3-HSP90 signaling axis diminished viral entry, while its activation promoted viral

entry (Fig. 6h-i, Extended Data Fig. 6d-f). Altogether, these data indicates that IL6-STAT3-HSP90 signaling axis mediates viral entry in aquatic ectotherms.

IL6-STAT3-HSP90 signaling axis mediates viral entry in endotherms

To determine whether IL6-STAT3-HSP90 signaling axis mediates viral entry in endotherms, which compared with ectotherms is evolutionarily less affected by environmental temperature, we firstly chose ubiquitous herpesvirus infection in mammals as examples. Lytic infection of murine gamma-herpesvirus 68 (MHV68) or human alpha-herpesvirus 1 (HSV-1), as exhibited by the expression of immediate early / early genes (ORF50 and ORF59 for MHV68, VP23 and VP26 for HSV-1), were accompanied with induced IL6 expression (Fig. 7a-d, Extended Data Fig. 7a-b). Besides that, treating cells with inhibitors or activators of IL6-STAT3-HSP90 signaling axis, markedly dampened or promoted the viral entry, respectively (Fig. 7e-f, Extended Data Fig. 7c-d), suggesting that IL6-STAT3-HSP90 signaling axis mediates herpesvirus entry in mammalian cells. Furthermore, as COVID-19 pandemic continues to be a major focus of global health concern, the effect of IL6-STAT3-HSP90 signaling axis on SARS-COV-2 infection was examined therewith. We prepared SARS-COV-2 pseudovirus by lentiviral vector-based transfection (Extended Data Fig. 7e), then infected 293T-ACE2 stable cell line with SARS-COV-2 pseudovirus. RT-PCR analysis showed that SARS-COV-2 pseudovirus infection increased the transcription of IL6 (Fig. 7g). Additionally, treating cells with inhibitors of IL6-STAT3-HSP90 signaling axis dampened the entry of SARS-COV-2 pseudovirus, respectively (Extended Data Fig. 7f). Similar result was observed for porcine epidemic diarrhea virus (PEDV), a member of coronavirus family infecting swine, that inhibitor treatment blocked the viral entry, while activator treatment promoted the viral entry (Fig. 7i, Extended Data Fig. 7g). Cell viability assay showed that all the drugs we used under our experimental conditions had a negligible cytotoxic effect on the cells (Extended Data Fig. 7h). Collectively, our data supports the conclusion that IL6-STAT3-HSP90 signaling axis mediates viral entry in endotherms.

Discussion

Viral diseases in aquaculture industry show typical seasonality and temperature dependency phenotype which causes billions of economic losses every year (Reverter et al., 2020). Here, by means of exploiting GCRV infection in grass carp as a model to dissect the molecular mechanism behind the phenomenon, we identified that IL6-STAT3-HSP90 signaling axis played an important role in GCRV entry. We found that temperature stress evoked a quick immune response, induced the activation of IL6-STAT3 signaling pathway and the expression of HSP90. Interestingly, HSP90 localized primarily on the plasma membrane and its expression was regulated by IL6-STAT3 signaling. As a key chaperon protein downstream IL6-STAT3 pathway, HSP90 interacted with VP7, a major outer capsid protein of GCRV, to induce a cytoskeletal rearrangement, morphological change, and induction of membrane receptor related genes, which collectively advanced the penetration of virus into host cells. Accordingly, exogenous expression of either IL6, HSP90, or VP7 in cells engendered an increased viral entry. Interestingly, we found that such a phenomenon might be universally conserved in the interplay between virus and host coevolution. Taken

together, we delineated the role of IL6-STAT3-HSP90 signaling axis exploited by viruses to benefit viral entry.

Virus-host-environment interaction is a theme that permeates the entire course of the coevolution of life, reflected not only by the arms race between viruses and their hosts, but also the cooperation whereby viruses contribute to cellular functions whereas cellular genes are picked up by viruses and employed for the essential viral functions such as replication or protein processing (Koonin and Dolja, 2013). Taking the environment into account, in natural systems, it is quite common that viral infection rate and symptomatic diseases are highly season dependent in livestock and aquaculture industries, from which viral diseases commonly prevail in summer, while diminish in winter (Pereiro et al., 2021; VanderWaal et al., 2017). However, little is known regarding how environmental temperature alter virus-host interactions. At this point, there are two ways to explain the phenomenon. The first is that both viral replication and host growth are highly temperature dependent. Higher temperature tends to boost within-host virus replication and promote viral transmission, while lower temperature keeps within-host virus accumulation low, suggesting the importance of seasonal dynamics on within-host virus accumulation as a key determinant factor (Honjo et al., 2020; Morin et al., 2013). On the other hand, the seasonal dynamics on host responses, such as the antiviral immune defenses and the metabolic rate, could be another determinant factor contributing the seasonality of viral diseases (Honjo et al., 2020; Moriyama et al., 2020). Yet, how seasonal temperature fluctuation impacts host responses and further shapes viral infection rate is still largely unknown. Our study utilized GCRV infection as a model to investigate the molecular mechanism of temperature dependent of aquatic viral diseases. The integrated data we provided strongly supports the hypothesis that temperature fluctuation might lead to imbalances of host innate immune responses, which further influence the within-host viral infection and transmission (Honjo et al., 2020). However, more in-depth study of the seasonality in viral dynamics and virus-host interactions in naturally occurring infections needs to be evaluated in future studies, aiming to provide new insights into the multifaceted cooperation of virus-host-environment coevolution, particularly in the context of rapid global climate change in the past decades.

The growth factor- or cytokine- induced STAT3 activation plays pleiotropic roles in oncogenesis, host defense, and homeostatic maintenance (Garg et al., 2021; Hillmer et al., 2016). Interestingly, both pro- and antiviral roles of STAT3 have been documented. These seemly contradictory responses appear to be dependent of virus, the physiological status of the cells involved, and the activation of distinct sets of target genes transcriptionally regulated by STAT3 (Kuchipudi, 2015). Notably, there is increasing evidence to suggest that STAT3 plays an important role in viral replication and pathogenesis, through a complex interplay between viruses and STAT3 signaling. For instance, STAT3 binds directly to viral genome to promote viral gene expression, aberrant activation of STAT3 induces the expression of antiapoptotic proteins, oncogenic proteins, and inflammation related proteins, which are proposed to be beneficial to the replication of viruses and pathogenesis (Chang et al., 2018; Harrison and Moseley, 2020; Kuchipudi, 2015). However, the precise role of STAT3 signaling in the early step of viral infection is not yet fully understood. To the best of our knowledge, our study is the first report showing that environmental temperature dependent IL6-STAT3 signaling could mediate virus entry, which has allowed us to extend

our understanding of the complex interplay between virus and IL6-STAT3 signaling. As the molecular mechanism, we found that HSP90, which is a ubiquitously expressed chaperon protein that contributes to the maintaining the proper folding, maturation, trafficking, stability, and activity of numerous client proteins (Geller et al., 2012), by itself is closely associated with STAT3, and can be regulated by IL6-STAT3-signaling. Our data suggested HSP90 is a pivotal chaperon protein for viral infection, and may function as a co-receptor for virus entry through binding directly with outer capsid proteins of GCRV. Our finding is consistent with plenty of laboratory studies that have shown that HSP90 is usually exploited by viruses to function as a receptor or co-receptor for viral entry (Lubkowska et al., 2021). Meanwhile, the notion that HSP90 may serve as a key molecule to mediate many viruses' entry raised several questions to be addressed. Given the broad range of viruses and viral client proteins HSP90 may target in general, the molecular mechanism in detail is far from clear. Whether a general mechanism of HSP90 mediating viral entry exists? Whether the ATPase activity of HSP90 is required to assist the process? Whether HSP90 promotes viral entry by converting subtle changes in conformation of bona fide viral receptors to switch their ligand binding affinity, or inducing and maintaining the post-translational modification of viral receptors for activation, resembling the examples in which HSP90 activates steroid hormone receptor and protein kinase cyclin-dependent kinase Cdk4 by inducing clients conformational change and phosphorylation, respectively (Pearl and Prodromou, 2006). More in-depth mechanistic study involving HSP90 mediated viral entry needs to be conducted to address these unanswered questions in future. Also, we do not exclude the possibility that other HSPs or cell membrane receptors participate in this process. As our transcriptomic and RT-PCR data from STAT3 deficient zebrafish and stattic treated cells showed that STAT3 also regulates many genes expression involving cell adhesion, cytoskeleton, and HSPs. Further in depth studies to dissect the precise role of STAT3 in mediating viral early infections could provide more valuable insights into viral pathogenesis. In addition, the activation of IL6-STAT3 signaling and HSP90 expression can be impacted not only by temperature fluctuation, but also other environmental factors/stimuli during the faming process of aquatic animals, such as ammonia nitrogen, dissolved oxygen, salinity, pH, and stocking density (Liang et al., 2020; Long et al., 2019). However, our understanding concerning how the environmental stress responses in aquaculture industry alters the interaction between pathogens and host immune system, as well as the further development of infectious diseases is quite incomplete. Nevertheless, our data uncovered a typical immune evasion strategy exploited by virus to benefit viral entry during early infection, providing critical mechanistic insight into the temperature dependent viral pathogenesis.

Temperature dependency of viral diseases in aquaculture industry is a prevailing phenomenon, which causes enormous economic losses worldwide every year (Pernet et al., 2012; Reverter et al., 2020). Our work suggested the molecular mechanism that temperature dependent IL6-STAT3-HSP90 signaling mediates viral entry, which might be universal in ectothermic fish, pinpointing an opportunity to develop innovative preventives to treat such diseases in aquaculture industry thereof. Furthermore, the conservativeness analysis showed that the IL6-STAT3-HSP90 signaling axis is highly conserved through evolution, from invertebrate insects to vertebrate mammals. Intriguingly, among all the viruses we tested, including RNA and DNA viruses, were all sensitive to inhibitors or activators of IL6-STAT3-HSP90

signaling axis during the early viral entry step. Interestingly, using lentiviral vector based SARS-COV-2 pseudovirus, we found that STAT3 or HSP90 inhibitors treatment blocked the entry of SARS-COV-2 pseudovirus as well, which is consistent with the findings from other groups showing that the propagation of SARS-COV-2 in host cells is dependent on IL6-STAT3 signaling and HSP90 expression (Li et al., 2020; Ulhaq and Soraya, 2020). Together with these observations, our work provides further credence to the pivotal roles of IL6-STAT3-HSP90 signaling axis mediating viral entry, expanding the functional repertoires of IL6 and HSP90 as canonical biomarkers for inflammation, cancer, and aging (Fuhrmann-Stroissnigg et al., 2018; Lindborg et al., 2015; Tanaka et al., 2014; Whitesell and Lindquist, 2005). Furthermore, our data together with recent findings from other groups suggest that IL6 and HSP90 may serve as risk factors of susceptibility to viral infection (Li et al., 2020; Lubkowska et al., 2021; Ulhaq and Soraya, 2020), highlighting the feasibility and rationality of developing targeted therapies towards IL6-STAT3 signaling and HSP90 for viral diseases in the future.

Declarations

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AUTHOR CONTRIBUTIONS

G.H., T.X., Y.Z., J.L. conceived this study and prepared the manuscript. G.H., Z.L., W.L., S.X., L.H., C.D., R.S., H.W., Y.Z., T.X., J.L. performed experiments and data analysis. All authors read and approved the manuscript.

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Figures

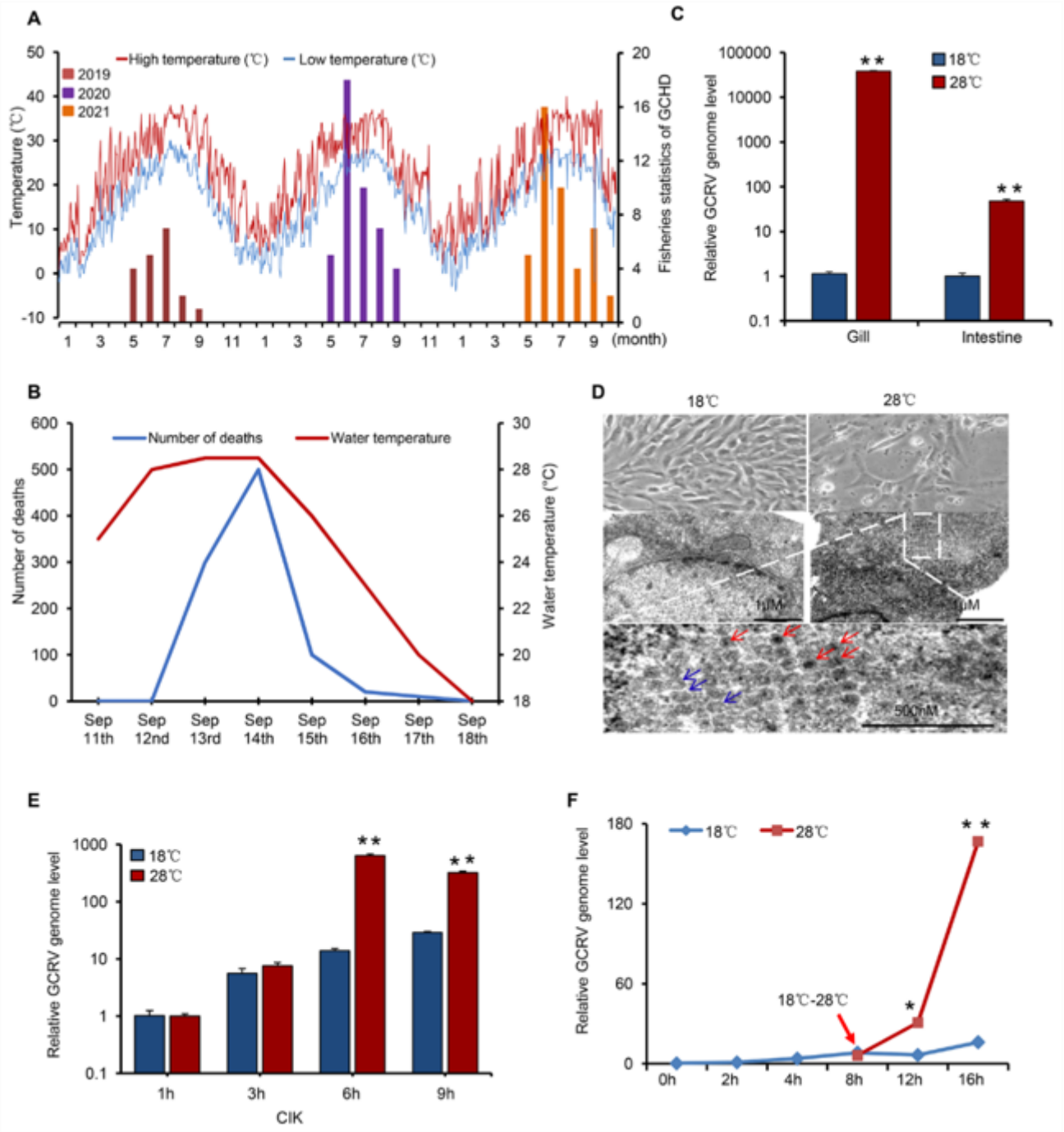

Figure 1

Temperature dependency of GCRV infection.(**a**) The seasonality of grass carp hemorrhagic disease was conducted based on a survey in 46 fisheries in Hunan province from 2019 to 2021. (**b**) Water temperature and deaths of grass carp hemorrhagic disease in fish ponds under natural environment were recorded. (**c**) The relative proliferation of GCRV genome in grass carp gill and intestine samples from (**b**) was analyzed by RT-PCR, in which 28 °C and 18 °C represented typical symtomatic infection on 14th September and asymtomatic infection on 20th September, respectively. (**d**) The Cytopathic Effect (CPE) and virion were observed by optical microscope and transmission electron microscope in CIK cells infected with GCRV under different temperature. (**e**) The relative GCRV genome replication from infected CIK cells under

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

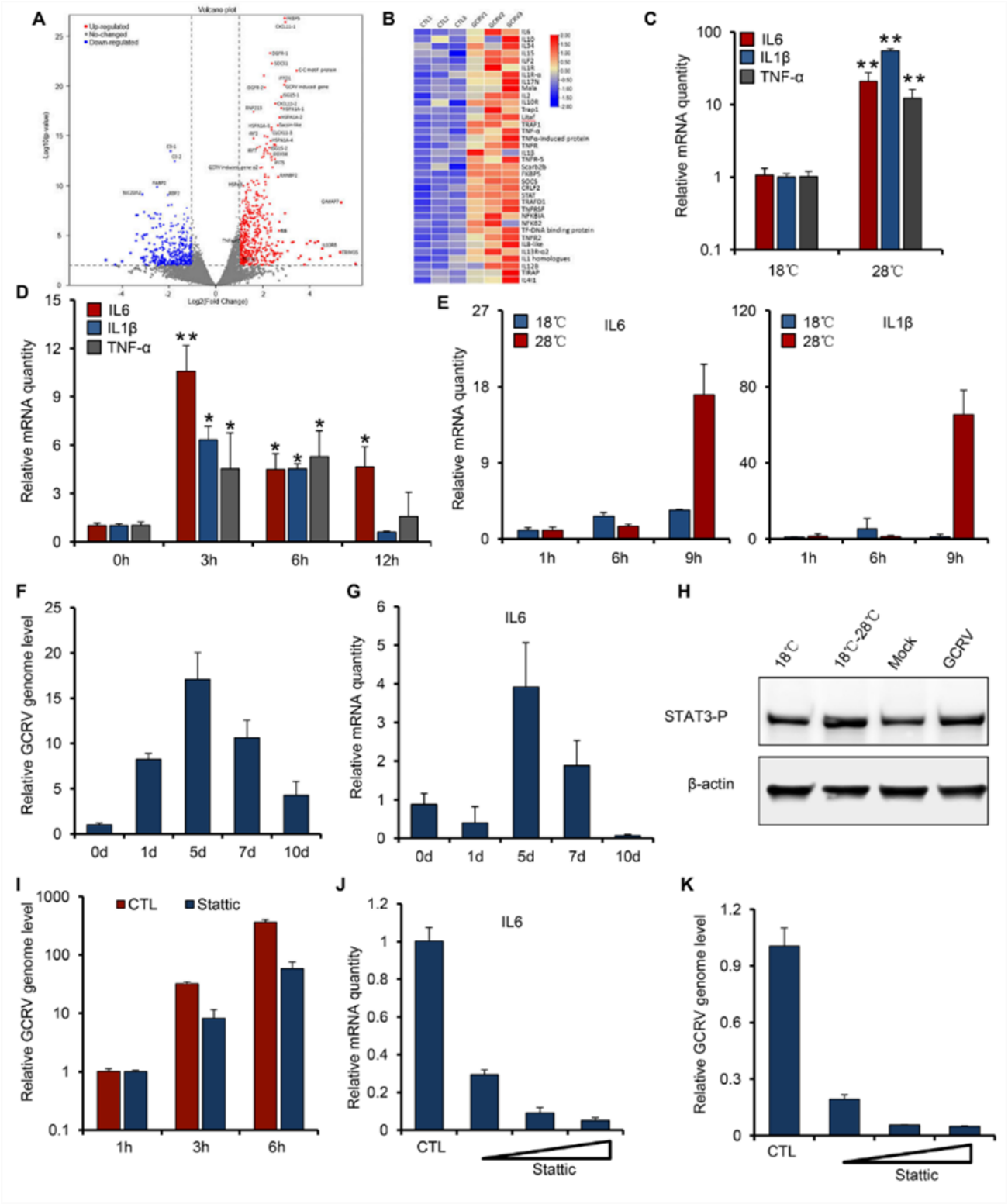

Figure 2

IL6-STAT3 signaling pathway mediates temperature dependent GCRV infection

(**a**) Transcriptomic data from GCRV infected spleen samples of grass carp were analyzed by volcano plot analysis. (**b**) Transcriptomic data from (**A**) profiling some upregulated proinflammatory genes were analyzed by heat map analysis. (**c**) The relative expression of proinflammatory genes from Fig. 1B-C in gills were analyzed by RT-PCR. (**d**) CIK cells under temperature stress from 18 °C to 28 °C were harvested to analyze the expression of proinflammatory genes by RT-PCR. (**e**) CIK cells infected with GCRV under different temperature were harvested to quantify the expression of proinflammatory genes by RT-PCR. (**f-g**) The relative proliferation of GCRV genome and expression of IL6 in spleen of grass carp from different timepoints were quantified by RT-PCR. (**h**) CIK cells under termperature stress or GCRV infection were harvested to analyze the IL6-STAT3 activation by western blotting analysis. (**i**) CIK cells pre-treated with STAT3 inhibitor stattic were infected with GCRV and harvested to analyze the relative viral genome level by RT-PCR. (**j-k**) CIK cells pre-treated with different dose of STAT3 inhibitor stattic were infected with GCRV and harvested to analyze the relative viral genome level by RT-PCR.

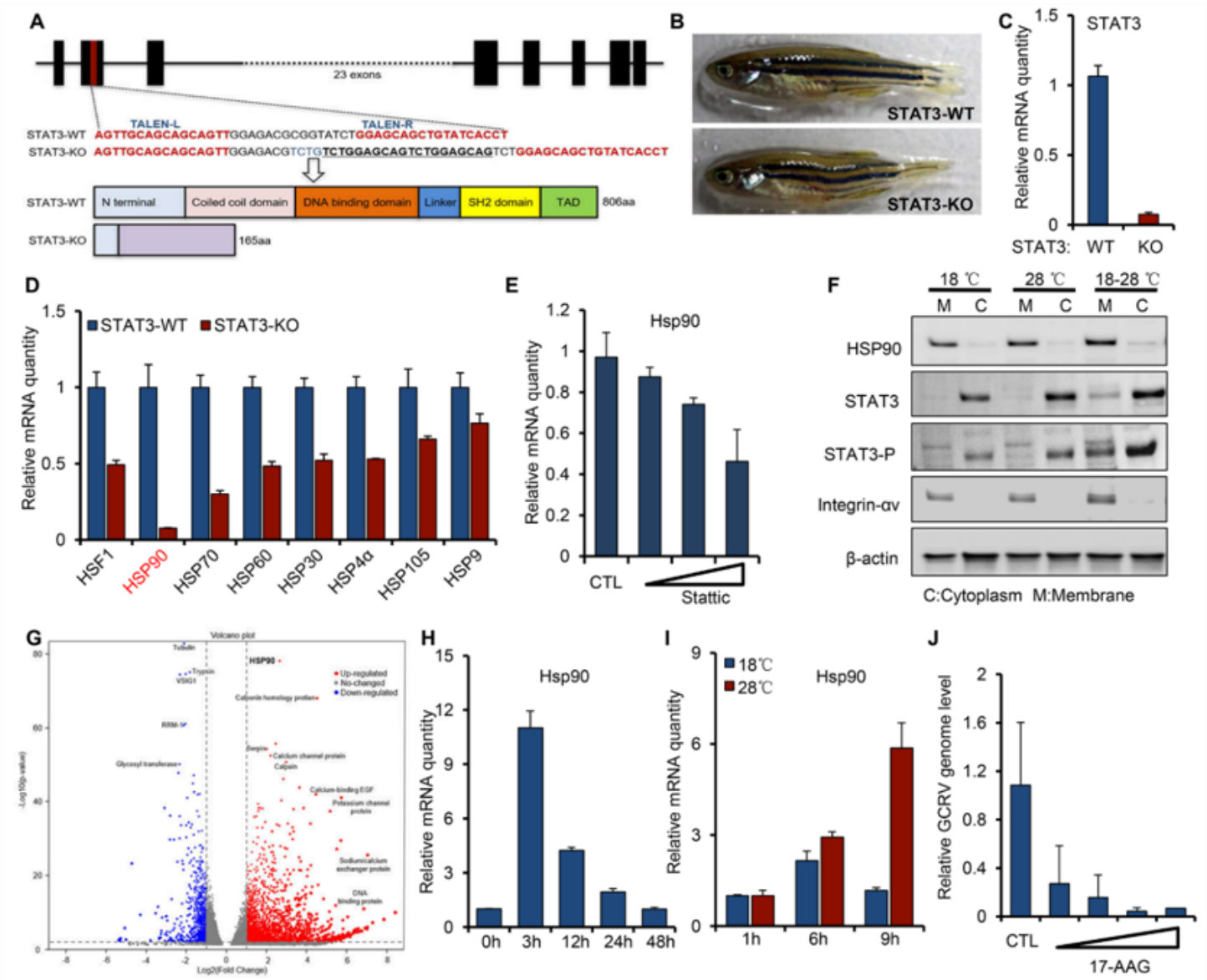

Figure 3

IL6-STAT3-HSP90 signaling axis mediates temperature dependency of GCRV infection.(**a-b**) Schematic diagram of constructing zebrafish STAT3 knockout mutant. (**c**) STAT3 gene expression in STAT3-KO mutant was verified by RT-PCR. (**d**) Transcriptomic data from STAT3-KO mutant were collected to quantify the relative expression of heat shock protein related genes. (**e**) CIK cells treated with different dose of STAT3 inhibitor stattic were prepared to quantify the relative expression of HSP90. (**f**) CIK cells under temperature stress were prepared to analyze the subcellular fractionation of HSP90 and STAT3 by western blotting. (**g**) Volcano plot analysis of all genes differentially regulated from transcriptomic data in CIK cells under temperature treatment from 18°C to 28°C. (**h**) Intestines of grass carp under temperature stress treatment were prepared to quantify the relative expression of HSP90. (**i**) CIK cells infected with GCRV under different temperature were prepared to analyze the relative expression of HSP90 by RT-PCR. (**j**) CIK cells pre-treated with different dose of HSP90 inhibitor 17-AAG were infected with GCRV and harvested to quantify the relative viral genome replication.

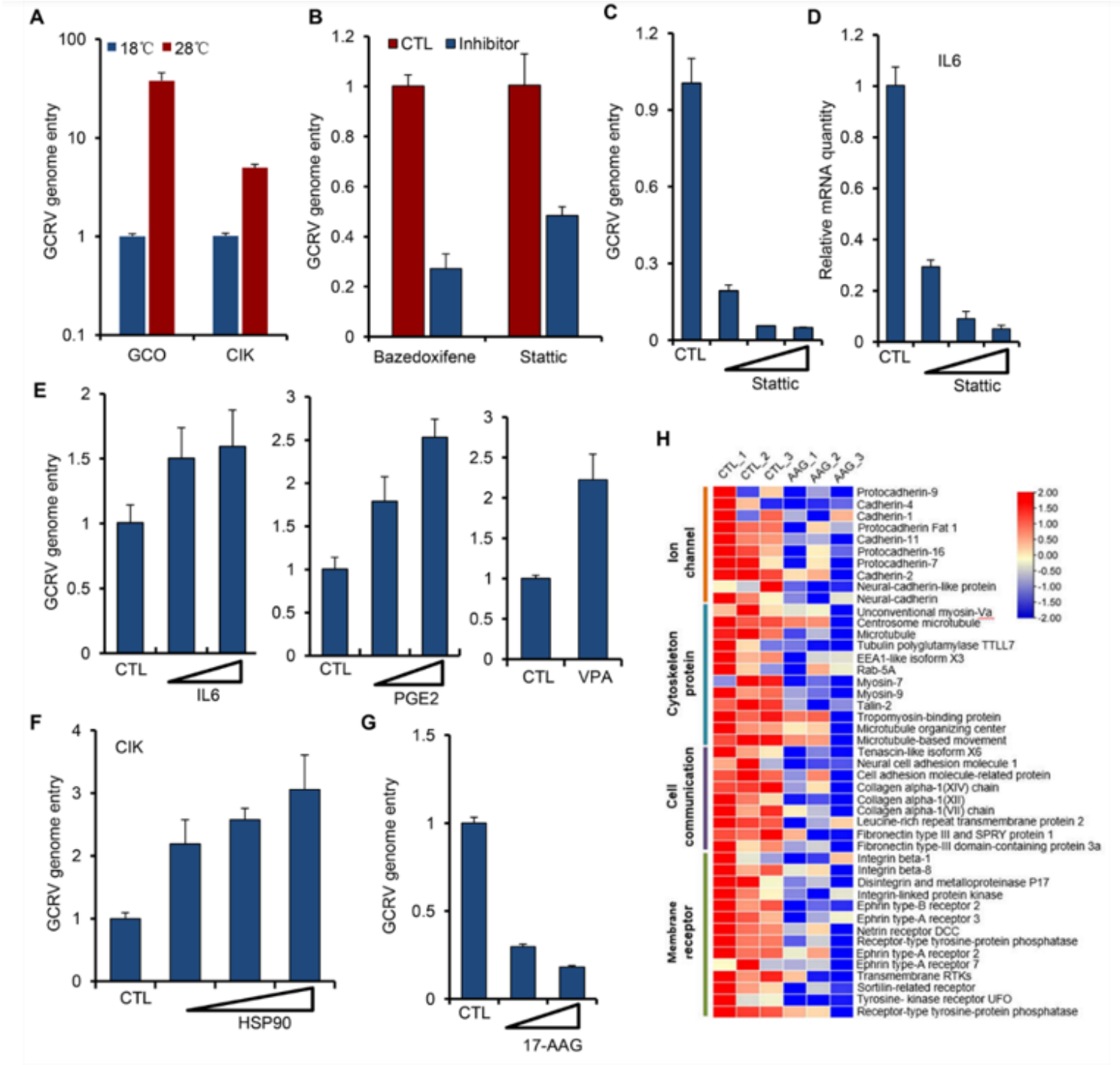

Figure 4

IL6-STAT3-HSP90 signaling axis regulates GCRV entry

(**a**) GCO/CIK cells infected with GCRV by 1h were prepared to quantify the relative genome entry level by RT-PCR. (**b**) CIK cells treated with gp130 or STAT3 inhibitor were prepared to quantify the relative GCRV genome entry level by RT-PCR. (**c-d**) CIK cells pre-treated with different dose of stattic were infected with GCRV for 1 h and harvested to analyze the relative viral genome entry level (**c**) and expression of IL6 (**d**) by RT-PCR analysis. (**e**) CIK cells pre-treated with different dose of IL6, PGE2, or VPA were infected with GCRV for 1 h and harvested to analyze the relative viral genome entry level by RT-PCR. (**f**) CIK 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.

(**g**) CIK cells pretreated with different dose of 17-AAG were infected with GCRV for 1 h and harvested to quantify the relative viral genome entry level by RT-PCR. (**h**) Transcriptomic data of CIK cells treated with 17-AAG were prepared to analyze the differentially downregulated genes by heat map analysis.

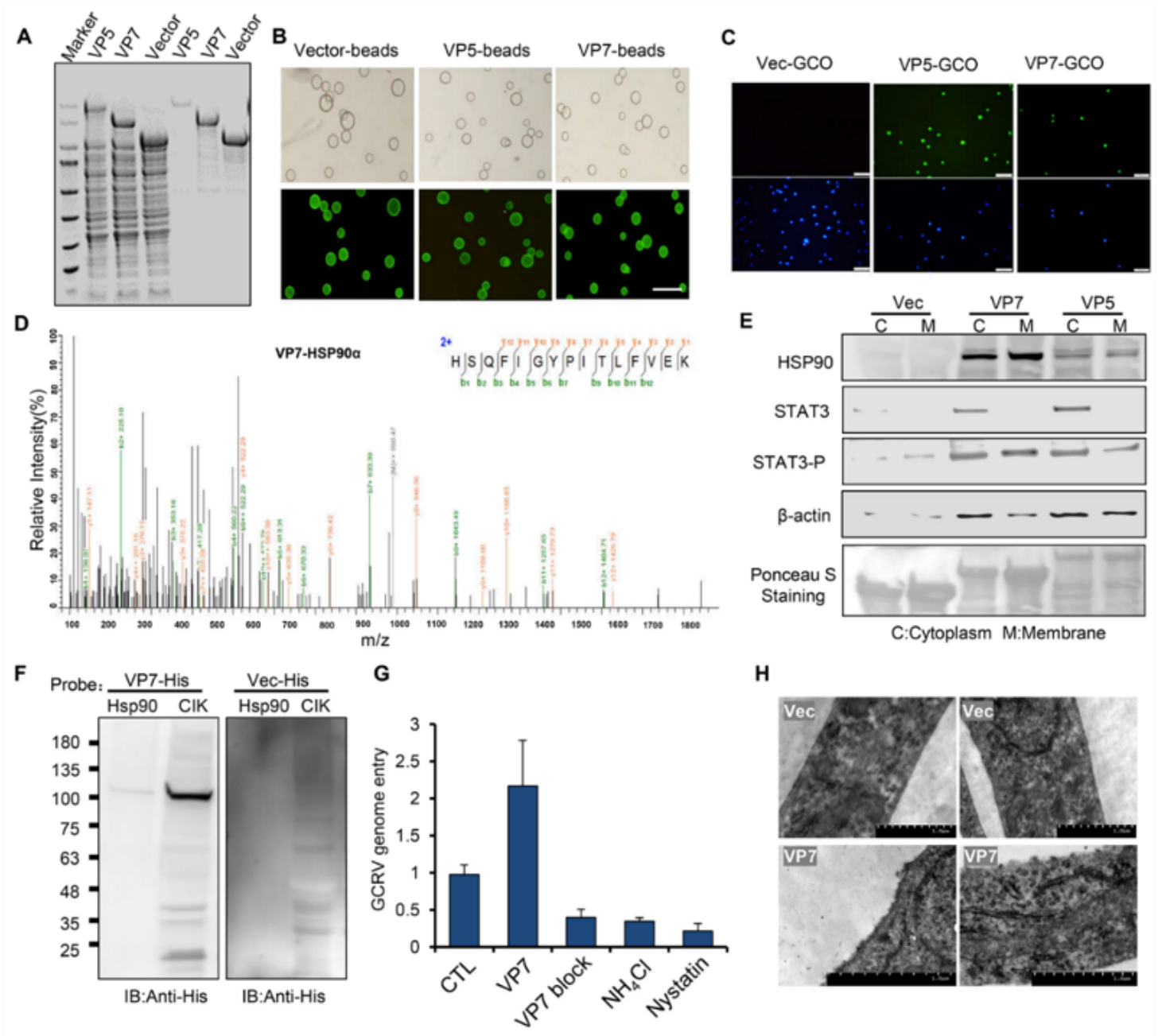

Figure 5

HSP90 interacts with VP7 and STAT3 on cellular membrane to promote GCRV entry

(**a**) Purified recombinant protein VP5-HIS-EGFP and VP7-HIS-EGFP to homogeneity were analyzed by SDS-PAGE. (**b**) Purified recombinant protein coupled with Ni-beads were examined by optical microscopy

analysis. Scale bars denotes 5 μm. (**c**) Binding of purified recombinant protein VP5-HIS-EGFP /VP7-HIS-EGFP to the plasma membrane surface of CIK cells were confirmed by optical microscopy analysis. Scale bars denotes 5 μm. (**d**) Mass spectrometry analysis of pulldown complex by purified VP7-HIS-EGFP identified HSP90 as a target of VP7. (**e**) Pulldown coupled with subcellular fractionation analysis were conducted to examined the interaction between VP7/VP5, HSP90 and STAT3.

(**f**) The direct interaction between VP7 and HSP90 was examined by far-western blotting analysis *in vitro*. (**g**) CIK cells pre-treated with VP7 or endocytosis pathway inhibitor were infected with GCRV for 1 h and prepared to quantify the relative viral genome entry by RT-PCR. Cells pre-treated with Vec or GCRV pre-treated with VP7 as two controls.

(**h**) CIK cells treated with purified VP7 protein were prepared to analyze the morphological and structural change by electron microscopy analyis.

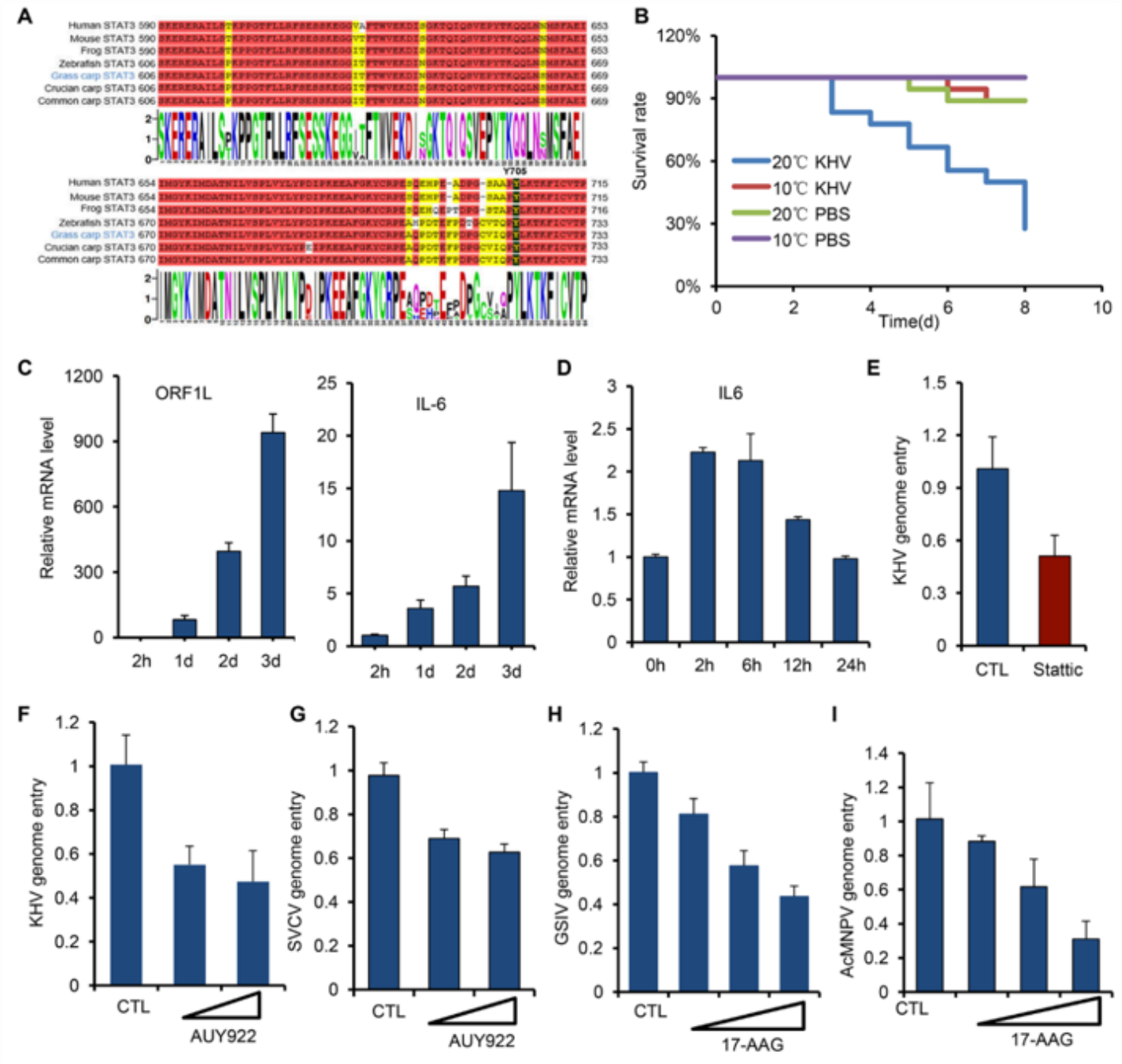

Figure 6

IL6-STAT3-HSP90 signaling axis mediates viral entry in ectotherms

(**a**) Evolutionary conserveness analysis of STAT3 was conducted by multiple amino acids alignment of STAT3 from different species. (**b**) Koi infected with KHV under different temperature were conducted for a survival analysis. (**c**) CCB cells under lytic infection of KHV were prepared to quantify the relative expression of ORF1L and IL6 by RT-PCR. (**d**) Gills of Koi under temperature stess at different timepoints were prepared to analyze the relative expression of IL6 by RT-PCR. (**e**) CCB cells pre-treated with stattic were infected with KHV for 1 h and prepared to analyze the relative genome entry level by RT-PCR. (**f**) CCB cells pre-treated with HSP90 inhibitor AUY922 were infected with KHV for 1 h and prepared to analyze the

relative viral genome entry by RT-PCR analysis. (**g-i**) EPC, GSM, or S9 cells pre-treated with different dose of HSP90 inhibitor were infected with SVCV, GSIV, and AcMNPV for 1 h, respectively. Cells were then prepared to quantify the relative viral genome entry level by RT-PCR.

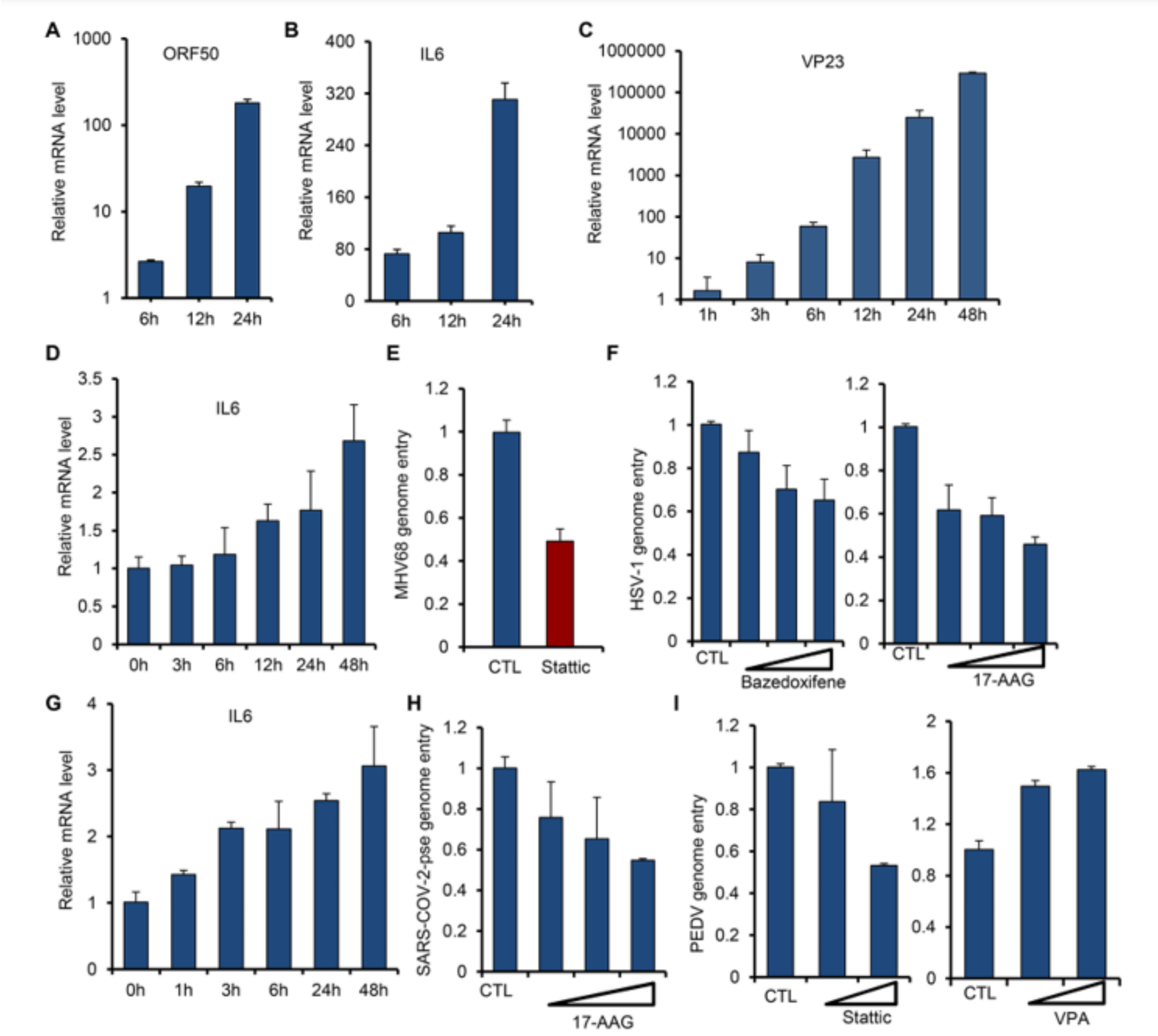

Figure 7

IL6-STAT3-HSP90 signaling axis mediates viral entry in endotherms

(**a-b**) MEF cells under lytic infection of MHV68 were harvested to determine the relative transcription of ORF50 (**A**) and IL6 (**B**) by RT-PCR. (**c-d**) 293T cells under lytic infection of HSV1 were harvested to determine the relative transcription of VP23 by RT-PCR. (**e**) MEF cells pre-treated with stattic were infected

with MHV68 for 1h and prepared to quantify the relative viral genome entry level by RT-PCR analysis. (**f**) 293T cells pre-treated with bazedoxifene or 17-AAG were infected with HSV1 for 1 h and prepared to quantify the relative viral genome entry by RT-PCR. (**g**) 293T-ACE2 cells infected with SARS-COV-2 pseudovirus at indicated timepoints were prepared to quantify the relative expression of IL6 by RT-PCR. (**h**) 293T-ACE2 cells pre-treated with 17-AAG were infected with SARS-COV-2 pseudovirus for 1 h and prepared to quantify the relative viral genome entry by RT-PCR. (**i**) PK cells pre-treated with stattic or VPA were infected with PEDV for 1 h and prepared to quantify the relative viral genome entry by RT-PCR.

Supplementary Files

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- Vector.avi
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