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NS38 is required for aquareovirus replication via interaction with viral core proteins and host eIF3A

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ABSTRACT

Aquareoviruses contain an 11-segmented double-stranded RNA genome. Previous studies indicated that NS38, a virus-encoded putative single-stranded RNA binding protein, interacts with NS80 in viral inclusion bodies (VIBs). However, the role of NS38 in aquareovirus infection remained unclear. Here, we found that NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) and the NS80-RNA complex in both transfected and infected cells. Knockdown of NS38 by siRNAs-115/219 clearly reduced viral infection, with decreased mRNA and protein yields. Moreover, NS38 can interact with host cellular eukaryotic translation initiation factor 3 subunit A (eIF3A) in transfected cells, while no association was detected between eIF3A and NS80. This study is the first to define that the NS38 is essential to viral replication. Together, our findings indicate that NS38 might function as a mediator by interacting with viral and host cellular components in VIBs during replication.

1. Introduction

Aquareoviruses, a genus of the *Reoviridae* family, comprise important pathogens in cultured aquatic animals (King et al., 2011). In particular, Grass carp reovirus (GCRV) is recognized as the most pathogenic among the isolated aquareoviruses (Rangel et al., 1999). GCRV contains a genome of 11 double-stranded (ds) RNA segments enclosed in a core surrounded with a double layered icosahedral capsid. The 11 genomic segments encode seven structural proteins (VP1–VP7) and five nonstructural proteins (NS80, NS38, NS31, NS26, and NS16) (Attoui et al., 2002; Zhang et al., 2010a).

Similar to other reoviruses, aquareovirus constitutes a nonenveloped icosahedral particle with an overall diameter of approximately 80 nm. The particle consists of two concentric protein shells, the outer-capsid and inner-capsid, which are assembled from the seven unique structural proteins. Previous genome characterization and three-dimensional (3D) reconstructions by Cryo-electron microscopy (Cryo-EM) demonstrated that the capsid proteins of GCRV share marked similarities with those of genus *Orthoreovirus* including mammalian reoviruses (MRVs) and avian mammalian reoviruses (Fang et al., 2005). VP1 has been shown to have guanylyltransferase transferase activity and RNA capping activities via S-adenosyl-L-methionine binding domains. VP2 (RNA dependent RNA polymerase, RdRp) and VP4 (nucleoside triphosphatase, NTPase) are able to form the RNA polymerase complex located at five-fold symmetry axes. The other inner-capsid proteins, VP3 and VP6, form the inner shell frame. These inner-capsid proteins play important roles in viral replication (Cheng et al., 2008, 2010; Wang et al., 2018; Yan et al., 2014) whereas the outer-capsid proteins VP5 and VP7 are required for virus-cell interaction and mediate virus entry during infection (Chen et al., 2018; Yan et al., 2015b; Zhang et al., 2018, 2010b).

In comparison, the nonstructural proteins are only observed in infected cells and play important role in viral replication. Our previous studies had shown that the nonstructural protein NS80 of aquareovirus could form viral inclusion bodies (VIBs, also termed viral factories or viroplasms) in singly expressed or infected cells, and also retained all the inner-capsid proteins in addition to NS38 within its inclusions (Fan et al., 2010; Shao et al., 2013; Yan et al., 2015a). Moreover, a short specific fragment in the C-terminal regions of NS80 were identified to be crucial for VIBs formation in infected cells, whereas the N-terminal regions of NS80 played important roles in interacting with viral proteins and supporting viral replication (Chen et al., 2016; Shao et al., 2013; Zhang et al., 2016). Other studies have also demonstrated that the nonstructural protein NS16 constitutes a fusion-associated small transmembrane protein and could induce cell-cell fusion in transfected and infected cells, with the nonstructural protein NS26 potentially utilizing the lysosomes to enhance the NS16-mediated fusogenic activity (Guo et al., 2015, 2013). Together, our previous studies indicated

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that viral nonstructural proteins play significant roles in aquareovirus replication.

Viruses lack biosynthetic capabilities; thus, they must rely on the host machinery for cell entry and self-protein synthesis to generate their progeny. Similar to other RNA and DNA viruses, reoviruses need to achieve cell-mediated inhibition of translation to effectively maintain viral protein synthesis after entry into cells. In the process, the reoviruses must alter the architecture of host cells to form an intracellular environment conducive to viral replication yet they also depend upon the host cell protein synthesis machinery to translate viral mRNAs into proteins (Desmet et al., 2014; Gratia et al., 2015; Walsh and Mohr, 2011). Toward this end, reoviruses encode proteins that directly interact with host translation initiation factors. For example, the rotavirus NSP3 protein binds eukaryotic translation initiation factor 4 subunit G (eIF4G) to mediate the selective translation of viral messages or modulate host protein synthesis (Gratia et al., 2016; Piron et al., 1998). MRV μNS and σNS proteins recruit eIF4E or eIF3A into VIBs to promote viral protein synthesis (Desmet et al., 2014). In addition, MRV infection can induce host phosphorylation of translation initiation factor eIF2a, which promotes the formation of discrete cytoplasmic inclusions termed stress granules (SGs). The σNS protein of MRV in VIBs, which interacts with μNS, can modulate SG protein localization by association with G3BP1 (Choudhury et al., 2017). Moreover, a recent study showed that ER markers and newly synthesized viral RNA were detected in viroplasms internal membranes (Tenorio et al., 2018). Together, this evidence indicates that σNS of MRV or its homologue in *Reoviridae* plays important roles to facilitate viral replication in the host cells through association with cell factors.

The nonstructural protein NS38 of aquareovirus constitutes a putative single-stranded RNA (ssRNA) binding protein that is retained within VIBs by interacting with NS80 (Shao et al., 2013). However, the roles of NS38 in the viral life cycle remained unclear. To better understand the detailed molecular mechanism of aquareovirus replication, the characteristics of NS38 in viral replication were investigated in this study. We found that NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) in addition to the NS80-RNA complex in both transfected and infected cells. Moreover, knockdown of NS38 by siRNAs-115/219 clearly inhibited viral yields at both mRNA and protein levels. Additionally, interaction between NS38 and eukaryotic translation initiation factor eIF3A was identified in transfected cells, suggesting that NS38 associates with host eIFs. The evidence provided in this study thus provides a foundation for further study of the molecular mechanisms of NS38 involvement in viral RNA replication and particle morphogenesis in host cells.

2. Materials and methods

2.1. Cells, virus, and plasmids

HEK 293T and Vero cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum(FBS) and 100 U/ml of penicillin and streptomycin (DMEM-10). CIK (*Ctenopharyngodon idellus* kidney) and FHM (*Fathead minnow*) cells were grown in minimum essential medium (MEM; Gibco-BRL) and medium 199 (M199; Gibco-BRL) supplemented with 10% FBS (MEM-10) respectively. Grass carp reovirus (strain GCRV-873), previously isolated and stored in the author's laboratory, was propagated in CIK cells with Eagle's MEM supplemented to contain 2% FBS (MEM-2) as previously described (Fang et al., 1989).

Plasmids used in this study (including pCI-neo-VP1, pCI-neo-VP2, pCI-neo-VP3, pCI-neo-VP4, pCI-neo-VP6, pCI-neo-NS38, pCI-neo-NS80, pCI-NS80(268–742), pCI-Flag-NS80, pGFP-NS38, pGFP-NSP5, and p-NS38-GFP-NSP5) were previously prepared and stored in our laboratory (Shao et al., 2013; Yan et al., 2015a; Zhang et al., 2016).

2.2. Antibodies and reagents

The polyclonal antibodies (pABs) of GCRV VP1, VP2, VP3, VP4, VP6, NS38, and NS80 were previously generated and stored in our laboratory (Fan et al., 2010; Shao et al., 2010, 2011; Wen et al., 2013; Yan et al., 2012). Mouse monoclonal IgG2b isotype control antibody was purchased from eBioscience Inc. (San Diego, CA). The anti-β-actin mouse monoclonal antibody (mAb), anti-GAPDH mouse mAb, anti-β-actin rabbit polyclonal antibody, and anti-eIF3A rabbit polyclonal antibodies were obtained from Proteintech (Wuhan, China). Anti-Flag mAb was the product of Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488 or 568 donkey anti-rabbit IgG (H + L) antibody, Alexa Fluor® 488 or 568 donkey anti-mouse IgG (H + L) antibody, Lipofectamine 2000, and Lipofectamine RNAi MAX were purchased from Invitrogen Co. (Carlsbad, CA). The Flag® Immunoprecipitation Kit was purchased from Sigma-Aldrich. Protease inhibitor cocktail and Pierce™ Co-Immunoprecipitation Kit (26149) were purchased from Thermo Fisher Scientific (Waltham, MA). RNase One was purchased from Promega (Madison, WI).

2.3. Infection assays

CIK cells were grown in 24-well tissue culture plates (Corning, Armonk, NY) at a density of 1×10^6 cells per well in MEM-10 prior to performing each experiment. The confluent cells were inoculated with GCRV at an MOI of 1 in serum-free MEM medium at 28 °C for 1 h. Following adsorption, cells were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.5) to remove non-adsorbed virions. Then, the infected cells were maintained in MEM-2 at 28 °C for 18 or 24 h, or different time points for various assays. Plaque assays were performed by determining the plaque formation unit according to a previously described method (Yan et al., 2015a).

2.4. Immunofluorescence (IF) assay

IF assays were performed as previously described (Shao et al., 2013). Briefly, for transfection analysis, Vero cells were selectively transfected with the indicated plasmids using Lipofectamine 2000 following the manufacturer's instructions and fixed at 24 h post-transfection (p.t.). For infection assay, CIK or FHM cells infected with GCRV were fixed in 4% paraformaldehyde at 18 h post-infection. After permeabilization by Triton X-100, all cells were incubated with specific primary antibodies followed by incubation with appropriate secondary antibodies. Then cells were washed extensively with PBS after each incubation step. DAPI staining was applied to detect the cell nucleus. All samples were observed using an Olympus-IX51 inverted microscope (Tokyo, Japan).

2.5. Co-immunoprecipitation (co-IP) assay and western blot (WB)

For co-IP experiments in mammalian cells, HEK 293T cells seeded in 25 cm^2 cell culture flasks (Corning) were transfected with various plasmids. Transfected cells were harvested at 24 h post-transfection and lysed in 600 μl IP lysis buffer containing protease inhibitor cocktail. Cellular debris was removed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Co-IP was conducted using the Thermo Scientific Pierce™ co-IP Kit according to the manufacturer's manual; briefly, the indicated antibody was first immobilized with AminoLink Plus coupling resin overnight, then the resin was washed and incubated with lysate by gentle mixing overnight at 4 °C. Subsequently, the resin was washed again and protein eluted using 80 μl elution buffer, then eluted with 20 μl of 5× SDS sample buffer by boiling for 10 min at 95 °C, followed by assessment of the samples using WB.

WB analysis was performed as previously described (Zhang et al., 2013). Briefly, whole-cell extracts were subjected to 10% SDS-PAGE

and transferred to PVDF membranes, followed by blocking with 5% nonfat milk in Tris-buffered saline-Tween (TBST) and probed with the indicated primary antibodies at 37 °C for 2 h. After washing with TBST, the membrane was incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Specific protein bands were developed by 5-bromo-4-chloro-3-indolylphosphate-nitro-blue tetrazolium.

2.6. RNase One treatment

HEK 293T cells were seeded in 25 cm^2 cell culture flasks prior to the day of transfection, then cells transfected with plasmids pCI-Vector, pCI-Flag-NS80 and pCI-Vector, or pCI-Flag-NS80 and pCI-NS38. Transfected cells were harvested at 24 h.p.t. and lysed in 800 μl IP lysis buffer containing protease inhibitor cocktail. Cellular debris was removed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Supernatants were transferred to tubes and incubated for 4 h at 4 °C with anti-Flag resin conjugate. CIK cells were infected with GCRV and lysed on ice with 1 ml lysis buffer when the cytopathic effect (CPE) was observed. For each IP, specific polyclonal antibodies that had been incubated with 40 μl of 1:1 slurry of Protein A beads (Invitrogen) and washed four times with lysis buffer were added to a 0.8 ml aliquot of lysate and then rotated for at least 4 h or overnight at 4 °C. Then, proteins (with anti-Flag resin conjugate with anti-NS80 resin conjugate) were split into two equal aliquots and resuspended in 45 μl of 10 mM Tris (pH 7.5). RNase One buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 200 mM sodium acetate) was added to a 1× final concentration to both aliquots. Then, 10 U RNase One was added to one aliquot and both samples were incubated at 37 °C for 30 min (Khorchid et al., 2002). After centrifugation at 1000 × *g*, supernatants were removed from the pellets and the resin was washed again and protein eluted using 40 μl elution buffer, then eluted with 10 μl of 5× SDS sample buffer by boiling for 10 min at 95 °C, after which the samples were assessed by WB.

2.7. RNA interference experiments and viral infectivity assay

Double-stranded siRNAs (siRNA-115, -219, -805) targeting NS38 and siRNA-control were designed and synthesized by Invitrogen Co. The sequences of NS38 siRNAs are listed in Table 1. RNA interference experiments were performed as previously described (Yan et al., 2015a). Briefly, CIK cells were transfected with 40 nM siRNA-control or 40 nM NS38 siRNAs, and then infected with GCRV at MOI of 1 at 6 h.p.t. Following incubation at 28 °C for 30 min, the inoculum was removed and rinsed three times with MEM, then subsequently incubated in 2% MEM. Infected cells were collected at 24 h post-infection and subjected to real time quantitative reverse transcription-PCR (qRT-PCR) and WB analysis. In addition, cell supernatant (extracellular virus) was harvested and virus titers were determined by plaque assays (Dobos, 1976).

2.8. Real time qRT-PCR

The real-time qRT-PCR primers designed by Primer Premiere software (Premier Biosoft International, Palo Alto, CA) are shown in Table 2. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following the protocols recommended by the manufacturer. qRT-PCR was performed on a real-time thermo cycler (Bio-Rad, Hercules, CA) using CFX 96 software. Each 20 μl qPCR reaction contained 1/5 μl reverse transcription sample and 10 μl iTaq Universal SYBY-Green Supermix plus 0.25 μM forward (F) and reverse (R) primers. Amplification was performed by denaturing at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Each reaction was performed in triplicate and β-actin was used as an internal control. The qRT-PCR data was analyzed using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001).

Table 1
Sequences of NS38 siRNAs.

Name	Sequence (5′–3′)
siRNA-115	CGUGAUCUUGUGUCUUCCGUGUACU AGUACACGGAAGACACAAGAUCACG
siRNA-219	CAAACCUGCUCACACCGCUACCAAU AUUGGUAGCGGUGUGAGCAGGUUUG
siRNA-805	CAUGUUCAGGCUGUGGACCCUUUCA UGAAAGGGUCCACAGCCUGAACAUG

Table 2
Primers used in RT-PCR.

Target	Sequence (5′–3′)	Product size
β-Actin	F: ATCGTGCGTGACATTAAGGAG R: GGAAGGAAGGCTGGAAGAG	135 bp
NS38	F: CTATGGCACTGGCGTTTA R: GTCGGGTAGTTCAGAGGG	209 bp
NS80	F: GGAAGCCGACAAGGGAATG R: TGGAGTAGCCGTGGGAAG	188 bp
VP1	F: TACCAACCCGTTAGTGCTT R: GGAGTAGTAGAATACCGTGGC	289 bp
VP2	F: TACGCCTACACCTTACTTCAA R: CGGTTCGGTCCACTCTATT	114 bp
VP3	F: GCTTTCTTCATCCGAGTGG R: GCGACGAGGACATTGGTA	242 bp
VP4	F: TGGCTCTATTGATGTCTGATG R: CAGTGATGTGGACGAAAGG	102 bp
VP6	F: CCCTGACTGGACGCCTAA R: CGCCTGCCACTTCTACGA	198 bp

2.9. Statistical analysis

Mean values were compared using the Student's *t*-test (GraphPad Prism 6, GraphPad Software, Inc., San Diego, CA). All graphs represent the means and standard deviations of normalized data points for triplicate samples from each representative experiment. Significant differences were determined using a two-tailed Student's *t*-test or a one-way ANOVA followed by a Tukey test for multiple comparisons (** $p < 0.01$).

3. Results

3.1. NS38 can interact with inner-capsid proteins (VP1–VP4 and VP6) in co-transfected cells

In previous studies, we have shown that aquareovirus NS38 and inner-capsid proteins VP1–VP4 and VP6 were retained within VIBs by interacting with NS80 (Shao et al., 2013; Yan et al., 2015a). In addition, using a rotavirus NSP5-based interaction analysis element (Contin et al., 2010), we constructed a protein-protein association platform and identified that the N-terminal region of aquareovirus NS80 is required for interacting with viral proteins during viral replication (Zhang et al., 2016). To determine whether interactions occur between NS38 and inner-capsid proteins (VP1–VP4 and VP6) during this process, the interactions between NS38 and these proteins were investigated by using a modified rotavirus-NSP5-based protein interaction system. Accordingly, Vero cells were co-transfected with NS38-GFP-NSP5 or GFP-NSP5 and plasmids expressing the respective inner-capsid protein. IF assays demonstrated that each of the inner-capsid proteins (VP1–VP4 and VP6) completely colocalized with NS38-GFP-NSP5. However, the distributions of viral core proteins VP1–4 and VP6 in cells co-transfected

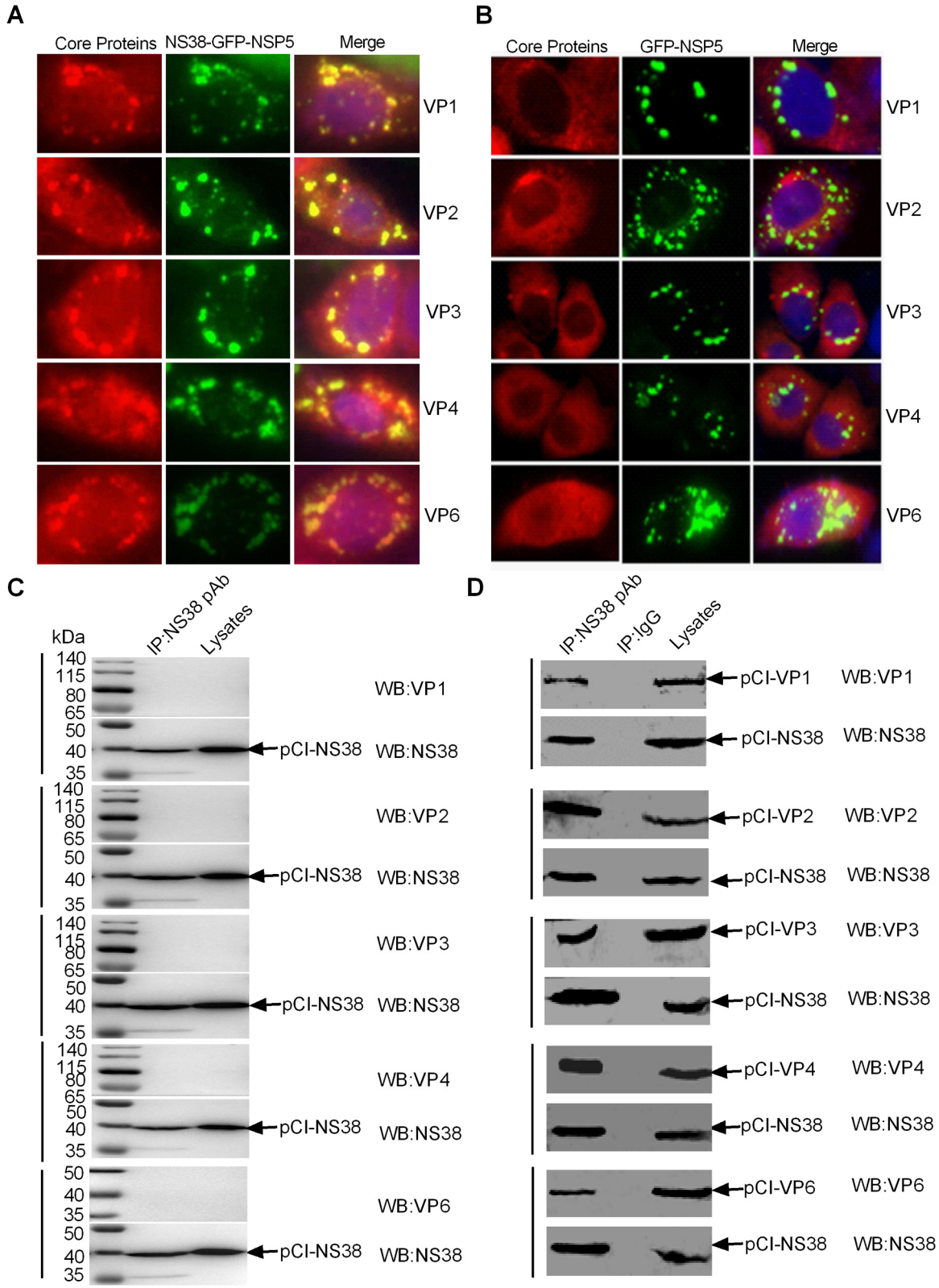

Fig. 1. NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) in transfected cells. (A and B) Vero cells were co-transfected with plasmids NS38-GFP-NSP5 or GFP-NSP5 and pCI-neo-VP1, pCI-neo-VP2, pCI-neo-VP3, pCI-neo-VP4, or pCI-neo-VP6, respectively. Cells were fixed at 24 h.p.t. and then stained with mouse polyclonal antibodies against VP1, VP2, VP3, VP4, or VP6 (red). Cell nuclei were counterstained with DAPI. (C) HEK 293T cells were transfected with plasmids pCI-neo-NS38. At 24 h.p.t., cells were harvested and lysed, then the samples were subjected to co-IP assays using anti-NS38 pAb. WB was probed with the indicated antibodies. (D) HEK 293T cells were co-transfected with plasmids pCI-neo-NS38 and pCI-neo-VP1, pCI-neo-VP2, pCI-neo-VP3, pCI-neo-VP4, or pCI-neo-VP6. At 24 h.p.t., cells were harvested and lysed, then the samples were subjected to co-IP assays using anti-NS38 pAb or control IgG. WB was probed with the indicated antibodies.

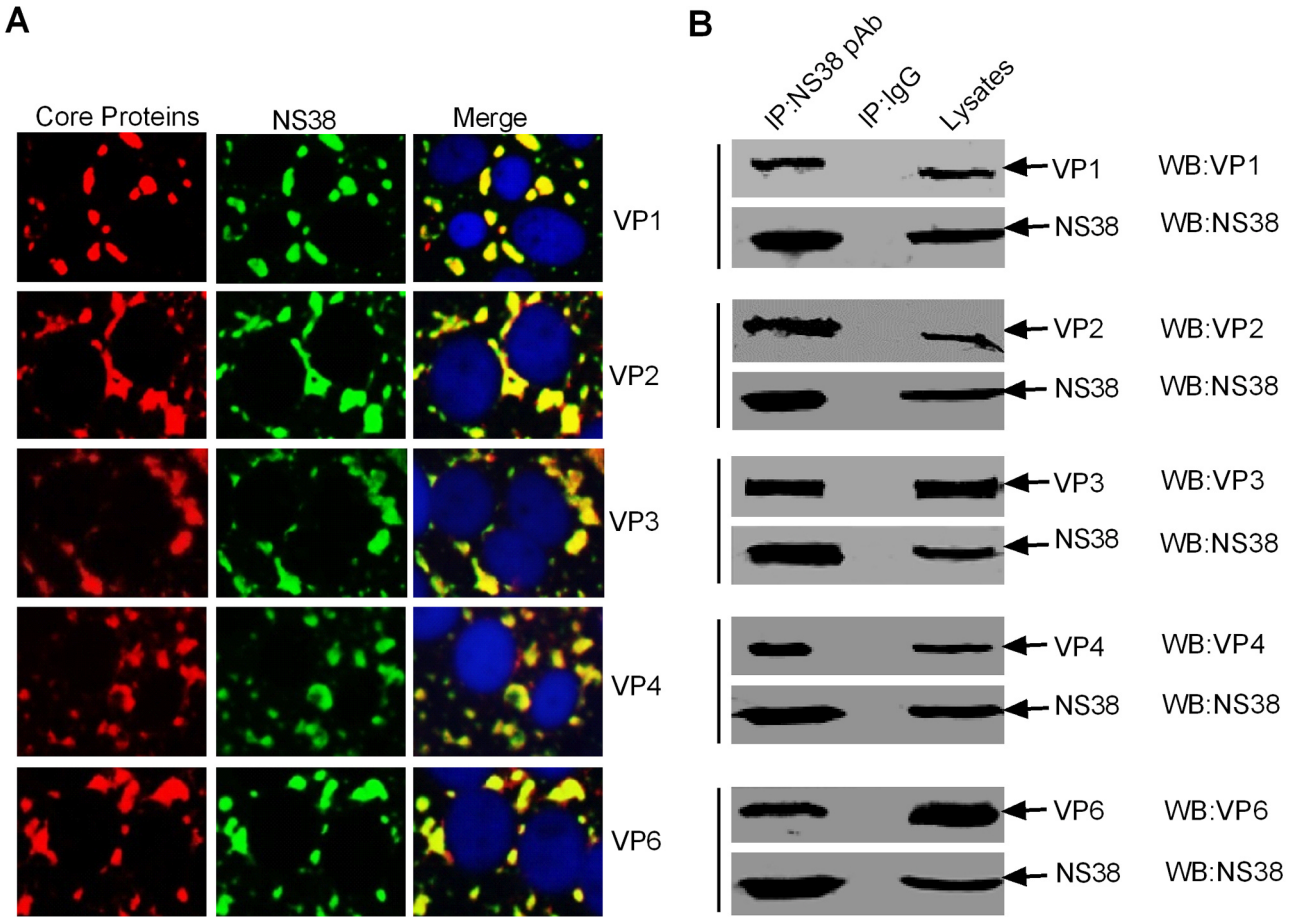

Fig. 2. NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) in infected cells. (A) CIK cells were infected with GCRV-873 at an MOI of 1, Cells were fixed at 18 h post-infection, then stained with rabbit polyclonal antibodies against NS38 (green) and mouse polyclonal antibodies against VP1, VP2, VP3, VP4, or VP6 (red). Cell nuclei were counterstained with DAPI. (B) CIK cells were infected with GCRV-873 at an MOI of 1, then cells were harvested and lysed at 18 h post-infection. The samples were then subjected to co-IP assays using indicated antibodies or control IgG. WB was probed with the indicated antibodies.

with GFP-NSP5 appeared in diffused pattern, no colocalizations were detected between them (Fig. 1A and B). For co-IP analysis to verify the interactions, the cells were co-transfected with pCI-neo-NS38 and each inner-capsid protein plasmid or transfected with pCI-neo-NS38 alone, followed by an NS38 IP and immunoblot with the VP1–VP4, VP6 antibodies. Consequently, the VP1–VP4 and VP6 proteins were each successfully immunoprecipitated by NS38 using an anti-NS38 antibody in co-transfected cells, while no cross-reacting was detected between NS38 and the core proteins with NS38 singly transfected cell lysate (Fig. 1C and D). These results suggested that NS38 interacts with all inner-capsid proteins in co-transfected cells.

To further exclude potential artifact or nonspecific binding in the interaction analysis between NS38 and core proteins, additional control experiments were also conducted in this study. Based on our previous report that NS80 residues aa 1–130 are necessary for interaction with NS38 (Zhang et al., 2016), the plasmid NS38-GFP-NSP5 was co-transfected either with a plasmid expressing full length NS80 (pCI-NS80) or an N-terminal truncated NS80 (pCI-NS80(268–742)) that was previously identified as having no interaction with NS38 (Zhang et al., 2016). As shown in Fig. S1A and B, NS38 could fully colocalize with full length NS80(1–742), whereas no overlapping signal was detected between NS38 and truncated NS80(268–742). To specialize the IF data, co-IP analysis was performed. As expected that NS38 could not immunoprecipitate with NS80(268–742) (Fig. S1C). Collectively, these results indicated that NS38 interacts with all inner-capsid proteins in co-transfected cells.

3.2. NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) during aquareovirus infection

To determine whether NS38 could colocalize with inner-capsid proteins during aquareovirus infection, CIK and FHM cells were infected with GCRV-873 for IF assays. It was found that NS38 colocalized with each of the inner-capsid proteins VP1–VP4 and VP6 within VIBs (Fig. 2A). To confirm the interaction between NS38 and viral core proteins, colocalization assays were also performed in FHM cells. As expected, NS38 signals fully overlapped with those of VP1–VP4 and VP6 in FHM cells (Fig. S2A). To verify the above results, the association between NS38 and each inner-capsid protein during infection was investigated by co-IP analysis. As shown in Fig. 2B, NS38 was efficiently pulled down along with VP1–VP4 or VP6. Moreover, all the interactions were also verified by reciprocal co-IP assays. These findings showed that each of the core proteins (VP1–VP4 and VP6) was efficiently co-immunoprecipitated with NS38 upon detection with an anti-NS38 pAb (Fig. S2B). Together, these results clearly indicated that NS38 interacts with all inner-capsid proteins in infected cells.

3.3. NS38 interacts with the NS80-RNA complex in infected and transfected cells

Studies of mammalian reovirus have shown that the μNS protein nucleates the VIBs, and that σNS localizes to the VIBs during infection through interacting with μNS (Miller et al., 2003). The σNS protein has

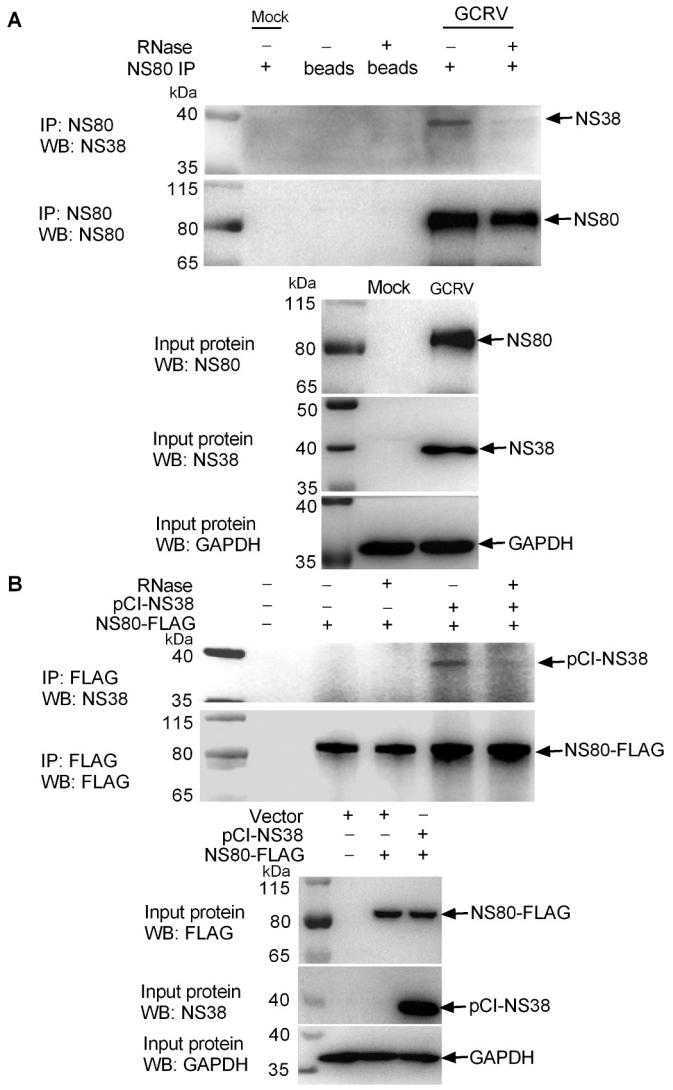

Fig. 3. NS38 interacts with the NS80-RNA complex. (A) Mock- or GCRV-infected CIK cells were lysed in non-denaturing buffer at 18 h post-infection and immunoprecipitated using NS80-specific rabbit polyclonal antiserum. IP with beads alone was used as a nonspecific binding control. The immunoprecipitated proteins were split into two samples that were either treated or not treated with 10 U of RNase One (Promega). Then, samples were subjected to WB analysis. (B) HEK 293T cells transfected with pCI-neo Vector, NS80-Flag with pCI-neo Vector, or both NS80-Flag with pCI-neNS38 were lysed in non-denaturing buffer at 18 h post-transfection and immunoprecipitated using NS80-specific rabbit polyclonal antiserum. IP with beads alone was used as a nonspecific binding control. The immunoprecipitated proteins were split into two samples that were either treated or not treated with 10 U of RNase One (Promega). Then samples were then subjected to WB analysis.

more recently been shown to play a role in ER tabulation (Tenorio et al., 2018). Similar to MRV σNS, the aquareovirus NS38 is a putative single-stranded RNA (ssRNA) binding protein, which has been identified to be retained within VIBs by associating with NS80 (Yan et al., 2015a; Zhang et al., 2016). To determine whether the NS80-associated NS38 is also complexed with RNA in infected cells, NS80 antiserum was used to co-IP NS38 from GCRV-infected CIK cell lysates. The immunoprecipitated proteins were then either left untreated or treated with RNase One to determine whether RNA digestion affected the NS38-NS80 association. It was found that the electrophoretic belt of NS38 in the RNase One-treated immunoprecipitates was obviously diminished in comparison to those in the untreated precipitates (Fig. 3A, upper). In contrast, similar amounts of NS80 were present in the treated and untreated immunoprecipitates (Fig. 3A, lower). This result suggested that NS38 might bind with NS80 through RNA association.

To determine whether the NS38 is able to form a complex with RNA in vitro, we co-transfected cells with plasmids pCI-neo Vector, NS80-Flag with pCI-neo Vector, or both NS80-Flag and pCI-NS38, then performed co-IP using the Flag-tag antiserum on the transfected cell lysates. Immunoprecipitates were either untreated or subsequently treated with RNase One. Fig. 3B shows that NS38 was found at expected amounts in the untreated immunoprecipitates but was obviously reduced in the precipitates treated with RNase One. The ability of NS38 to be co-immunoprecipitated with NS80 in RNase One-untreated samples from transfected cells suggested that the interaction between NS38 and NS80 might partly depend on RNA as an intermediate. Together, these results indicated that RNA contributes to the NS38 association with NS80.

3.4. Knockdown of NS38 reduces the expression of NS80 and core structural proteins

As NS38 could interact with all of the core structural proteins in transfected and infected cells, it may, as for NS80, be critical during virus replication and assembly. To determine whether NS38 is required for viral replication, three different NS38-specific siRNAs (siRNA-115, siRNA-219, and siRNA-805) plus control-siRNA were synthesized and co-transfected with NS38 plasmid into HEK293T cells, after which cells were harvested at 24 h.p.t. The relative expression levels of NS38 were strongly diminished with siRNA-115 and siRNA-219, whereas no obvious influence was detected with siRNA-805 (Fig. 4A and B). To evaluate whether the three different NS38-specific siRNAs had an effect on NS80 and five core proteins expression, CIK cells were transfected with each siRNA as described above and infected with GCRV at MOI of 1, then subjected to WB analysis. It showed that knockdown of NS38 with siRNA-115 or -219 clearly reduced the expression of NS38, NS80 and five core structural proteins (Fig. 4C). To confirm the role of NS38 in viral infection, viral plaque assays were also conducted. As shown in Fig. 4D, the virus titers were decreased by nearly 4 log10 steps by knockdown of NS38 with siRNA-115 or -219, compared with those of mock-treated GCRV-infected cells, whereas no effect was observed with control-siRNA or siRNA-805, indicating that expression of all the viral structural proteins could be affected by knockdown of NS38. Further statistical analysis showed that knockdown of NS38 by siRNAs-115 and 219 markedly lowered the expression of all the detected proteins to < 40% that of control-siRNA (Fig. 4E). Notably, the expression level of VP2 and VP4, which comprise the RNA polymerase complex during viral infection, was markedly decreased by siRNA-115 or -219 (Fig. 4E). To understand whether NS38 knockdown yielded a similar effect at the mRNA level, whole-cell lysates from siRNA-transfected and GCRV-infected cells were examined by qRT-PCR. The relative mRNA transcription levels of all the core structural proteins were reduced by over 50% when NS38 was knocked down by siRNA-115 or siRNA-219 (Fig. 4F), which corresponded with the trends of reduced protein expression. Taken together, these results clearly indicated that knockdown of NS38 by siRNA-115 or siRNA-219 reduced NS80, core protein expression and virus replication.

3.5. NS38 can interact with host eIF3A in transfected cells

The nonstructural protein σNS of mammalian orthoreoviruses strongly interacted with eIF3A and pS6R in the 43S preinitiation complex, suggesting that σNS recruits or maintains ribosomes within VIBs to facilitate viral translation (Desmet et al., 2014). To assess the potential associations between host eIFs and NS38 or NS80, Vero cells were co-transfected with eIF3A and NS38 or NS80, and fixed at 18 h post-transfection for IF analysis by using eIF3A, NS38 or NS80 pAb, respectively. It was observed that NS38 could colocalize with eIF3A, whereas no specific colocalization was detected between NS80 and

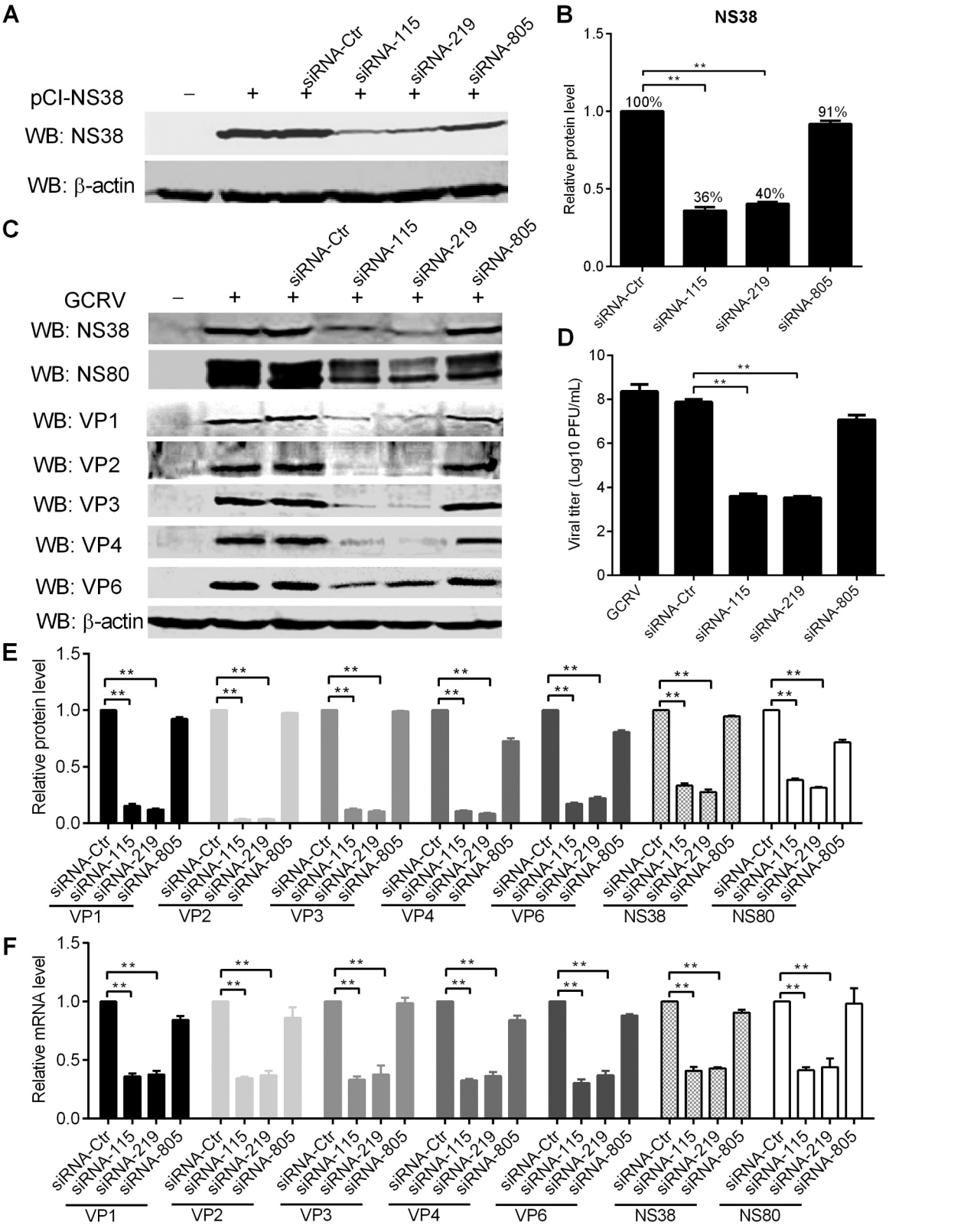

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Fig. 4. Knockdown of NS38 impairs NS80, core proteins expression and virus replication. (A) HEK 293T cells were co-transfected with control-siRNA or NS38 siRNAs (siRNA-115, siRNA-219, or siRNA-805) and pCI-neo-NS38, cells were analyzed by WB at 24 h.p.t. (B) Relative protein expression levels of NS38 were normalized to that of β-actin and then evaluated to maximum value. Results were obtained from three independent experiments. (C) CIK cells transfected without (mock) or with control-siRNA or NS38 siRNAs (siRNA-115, siRNA-219, or siRNA-805). At 6 h.p.t., CIK cells were infected with GCRV-873 at an MOI of 1, then cells were harvested at the indicated time points after infection and subjected to WB analysis for the expression of NS38, NS80 and inner-capsid proteins (VP1–VP4 and VP6) using the indicated antibodies. (D) CIK cells transfected without (mock) or with control-siRNA or NS38 siRNAs (siRNA-115, siRNA-219, or siRNA-805). At 6 h.p.t., CIK cells were infected with GCRV-873 at an MOI of 1, then culture supernatants were harvested at the indicated time points after infection and subjected to viral plaque assay. (E) Relative protein expression levels of NS38, NS80 and inner-capsid proteins (VP1–VP4 and VP6) were normalized to that of β-actin and then evaluated to maximum value. Results were obtained from three independent experiments. (F) CIK cells transfected with control-siRNA or NS38 siRNAs (siRNA-115, siRNA-219, or siRNA-805). At 6 h post-transfection, CIK cells were infected with GCRV-873 at an MOI of 1. Cells were collected at 18 h post-infection and total RNA was extracted from cells using TRIzol reagent. The relative mRNA levels of NS38, NS80 and inner-capsid proteins (VP1–VP4 and VP6) were analyzed by qRT-PCR. The data represent the means plus standard deviations for three independent experiments and were tested for statistical significance. $^{**}p < 0.01$.

eIF3A (Fig. 5A). To confirm interactions between eIF3A and NS38 or NS80, co-IP analyses were further performed. NS38 was efficiently pulled down with eIF3A (Fig. 5B), whereas no interaction was observed between NS80 and eIF3A (Fig. 5C), suggesting that NS38 is required to interact with host eIF3A. Together, these results indicated that NS38 could interact with host eIF3A and is involved in viral mRNA translation during infection.

4. Discussion

The replication and assembly of many viruses take place in viral factories during infection (Heath et al., 2001; Novoa et al., 2005; Wileman, 2006). Numerous studies have demonstrated that MRV and rotavirus generate VIBs in the cytoplasm during infection, with viral proteins, newly synthesized RNA, and nascent viral particles being retained within these particular structures (Dales, 1965; Eaton et al., 1987; McNulty et al., 1976; Sharpe et al., 1982; Shimizu et al., 2009; Spendlove et al., 1963). Moreover, the σNS protein was required for nucleation of viral assembly complexes in VIBs by associating with μNS and cellular translation-related factors for accomplishing their replication cycle (Becker et al., 2001). Additionally, we have recently characterized the functions, interacting proteins (including NS38), and critical domains of aquareovirus NS80 during infection (Shao et al., 2013; Yan et al., 2015a; Zhang et al., 2016). In the present study, we focused on the role of NS38 in the aquareovirus life cycle and demonstrated that NS38 plays a crucial role in viral replication and assembly.

Using a rota-NSP5 based protein interaction platform previously established in our laboratory, we first identified that NS38 interacts with inner-capsid proteins (VP1–VP4 and VP6) in transfected cells. Then, we confirmed that each interaction occurred in infected cells. We showed the importance of NS38 in viral replication by assessing the effects of its depletion using RNA interference, which revealed that NS38 knockdown not only decreased NS80 expression but also strongly diminished viral core structural protein synthesis. Notably, we found that the expression of RNA polymerase complex proteins VP2 and VP4 was obviously reduced by knockdown of NS38 compared to that of the

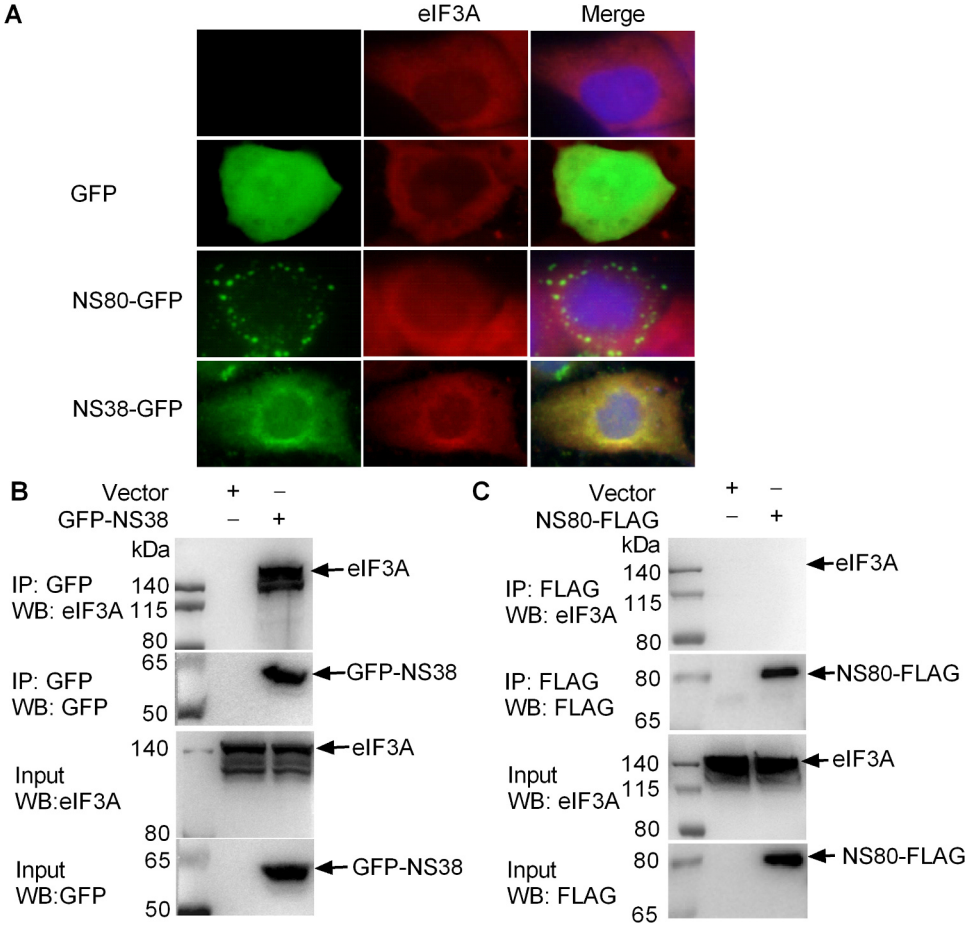

Fig. 5. NS38 can interact with host eIF3A in transfected cells. (A) Vero cells were transfected with plasmids GFP-Vector, GFP-NS38 or GFP-NS80 respectively. Cells were fixed at 24 h.p.t. and then stained with rabbit polyclonal antibodies against eIF3A (red). Cell nuclei were counterstained with DAPI. (B and C) HEK 293T cells were transfected with plasmid GFP-NS38 or Flag-NS80. At 24 h.p.t., cells were harvested and lysed, the samples were then subjected to co-IP assays using anti-GFP pAb or anti-Flag mAb. WB was probed with the indicated antibodies.

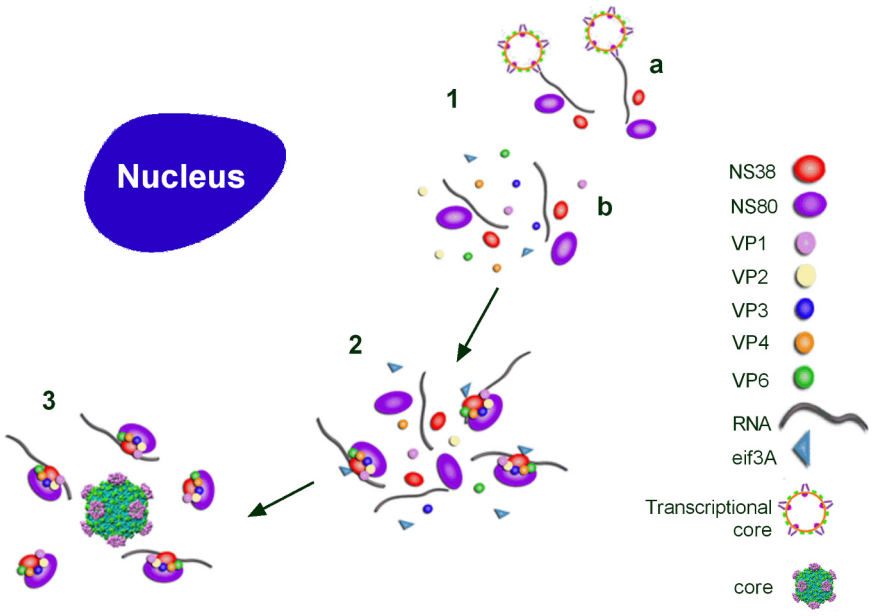

Fig. 6. Model of NS38 association with viral core components and cellular eIF3A in aquareovirus replication and assembly. Step 1. Newly synthesized viral mRNAs in transcriptionally active core particles are released from turret protein VP1 into cytoplasm, and then synthesis of NS38 and NS80 (a), and core structural proteins (b). Step 2. NS38 interaction with viral core proteins and cellular component eIF3A. Step 3. Nascent core assembly in mature VIBs.

other three core frame proteins VP1, VP3, and VP6. The results were further confirmed by determining actual mRNA transcription levels using qRT-PCR. Given that the proteins VP2 and VP4 are components of the RNA replicase complex (Wang et al., 2018), the observation that knockdown of NS38 markedly decreased viral yields might be associated with directly reduced expression level of these proteins, which in turn would affect viral RNA synthesis activity. In addition, the reduced core frame protein expression may lead to low progeny particle assembly. Excepting the association of NS38 with NS80 and core proteins, we are currently working on the identification between NS38 and outer-capsid proteins (VP5 and VP7) or aquareovirus three non-structural protein NS31, NS26 and NS16 during aquareovirus replication and assembly. Although the interaction mechanism between NS38 and viral protein is unclear, role of NS38 played in viral replication is obvious. Taken together, these results indicated that NS38 constitutes a critical factor for viral replication and particle assembly.

Similar to σNS in MRV, NS38 is considered to have ssRNA-binding ability and thought to be involved in viral proteins synthesis. To address whether NS38 possesses RNA binding properties, the role of RNA was determined by analyzing the effect of RNase treatment on the NS38 and NS80 association. Our results showed that NS38 strongly associated with the NS80-RNA complex in immunoprecipitated complexes from infected (Fig. 3A) and transfected cells (Fig. 3B) when lysates were generated without RNase One treatment. Conversely, only a weak NS38 band could be detected when cell lysates were treated with RNase One, suggesting that a small number of RNAs remained in the cell lysates to associate with NS38 even following this treatment. The observation that NS38 was bound to RNA in transfected cells might be a consequence of binding to cellular RNA, because MRV σNS functions as an ssRNA-binding protein that is isolated from infected cells in large RNA-containing complexes, and binds RNA in a sequence-independent manner in vitro. In particular, the N-terminal 11 residues of σNS have been previously shown to be required for both optimal ssRNA binding and formation of the large σNS-RNA complexes (Miller et al., 2003). The nature of NS38 co-immunoprecipitated with RNAs in vitro may thus be related to its RNA binding activity.

To be efficiently replicated in host cells, viruses must evolve to encode a protein to promote their own translation by recruiting host translational factors to the sites of viral replication as is suggested for DNA viruses, such as poxviruses and asfarviruses, which replicate in the cytosol (Castello et al., 2009; Katsafanas and Moss, 2007; Walsh et al., 2008). In turn, non-enveloped RNA viruses such as mammalian reoviruses can compartmentalize the translational machinery within virus-induced VIBs (Tenorio et al., 2018). eIF3A is essential for most forms of cap-dependent and cap-independent translation initiation in host cells and has also been identified to be involved in GCRV infection (Desmet et al., 2014; Guo et al., 2017). Considering that NS38 is a second main component in inclusion structures excepting NS80 and exhibits ssRNA binding features, we chose to assess possible interaction between host eIF3A and NS38 or NS80. The IF and co-IP results showed that eIF3A could be recruited into viral factories by interacting with NS38; however, no interaction was detected between eIF3A and NS80. This evidence strongly suggested that NS38 may play an important role in viral protein synthesis. Based on the results presented in this study and combining our previous findings (Shao et al., 2013; Yan et al., 2015a), we proposed that NS38 is required for aquareovirus replication via interaction with viral and host cellular component eIF3A. As schematically illustrated in Fig. 6, 1) Newly synthesized viral mRNAs in transcriptionally active core particles are released from turret protein VP1 into cytoplasm following viral infection, and then first synthesis of viral nonstructural protein NS38 and NS80(a), and second conducting viral core structural proteins expression (b). 2) NS38 interaction with newly synthesized viral core proteins and adjacent cellular component eIF3A at the site of inmature VIBs. 3) Nascent core assembly at the site of mature VIBs, which formed by NS80, with assistance of NS38.

The active translation of many viruses occurs within viral factories and host translational factors are also compartmentalized within viral inclusions (Desmet et al., 2014; Heath et al., 2001; Novoa et al., 2005; Wileman, 2006, 2007). The findings that knockdown of NS38 reduced both the protein and mRNA expression associated with viral infection, along with the interaction of NS38 with eIF3A in VIBs during aquareovirus infection, indicated that NS38 facilitates important steps in aquareovirus replication by interacting with other viral proteins and cellular translational components. It is possible that the effects of NS38 on viral protein synthesis are mainly due to its RNA binding characteristics to facilitate interactions with host cell translational factors for viral replication, as has been reported for rotavirus NSP3 and MRV σNS (Desmet et al., 2014; Piron et al., 1998). The function of NS38 and host translation factors in viral replication and assembly thus needs to be intensively investigated in future studies.

In summary, aquareovirus NS38 was found to interact with five viral core structural proteins, RNA, and host eIF3A during replication for efficient viral protein synthesis. In addition, knockdown of NS38 expression by N-terminal targeting siRNAs significantly reduced the

syntheses of NS80 and core proteins, particularly RNA polymerase complex proteins VP2 and VP4, along with viral infection. To our knowledge, this is the first report to indicate that NS38 plays an indispensable role in viral replication and assembly. The results provided in this study lay a foundation for further elucidating the role of NS38 in aquareovirus replication and pathogenesis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.01.029.

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