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Duck karyopherin α4 (duKPNA4) is involved in type I interferon expression and the antiviral response against Japanese encephalitis virus

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ABSTRACT
Karyopherin α4 (KPNA4) is an adaptor molecule that mediates type I interferon (IFN) production by facilitating the nuclear translocation of IFN transcription factors. Here, we cloned the duck KPNA4 (duKPNA4) gene and analyzed its involvement in type I IFN expression as well as antiviral response against Japanese encephalitis virus (JEV). The full-length duKPNA4 gene encoded a 520-amino acid protein that shared 97.3–98.7% sequence similarity with its orthologues in chickens, humans and mice. The duKPNA4 was extensively expressed in various duck tissues at the mRNA level. Analysis of the subcellular localization of duKPNA4 by immunofluorescence assays indicated that the duKPNA4 was primarily distributed in both the cytoplasm and nucleus in primary duck embryonic fibroblasts (DEFs). However, it translocated from the cytoplasm to the nucleus in response to poly(I:C) stimulation or JEV infection. The duKPNA4 interacted with duck IFN regulatory factor 7 and facilitated its nuclear translocation, thereby up-regulating the expression of IFN-α and IFN-β induced by JEV infection and inhibited JEV replication in DEFs. These data demonstrate the importance of duKPNA4 in type I IFN signaling as well as the antiviral response against JEV replication.

1. Introduction
Karyopherin (KPNA), also known as importin α, acts as an adaptor molecule between nuclear-localization-sequence (NLS)-bearing cargo proteins and importin β (Goldfarb et al., 2004), and plays an essential role in the active transport of proteins from the cytoplasm to the nucleus (Smith et al., 2018). In eukaryotic cells, macromolecules (≥ 25 nm in diameter or ≥ 40 kDa) bearing an NLS can be translocated from the cytoplasm into the nucleus by importin α/β heterodimers (Fagerlund et al., 2005).

In eukaryotes, multiple subtypes of KPNA have been identified. Mice and humans have six (KPNA1, 2, 3, 4, 5 and 7) and seven (KPNA1, 2, 3, 4, 5, 6 and 7) subtypes, respectively, whereas budding yeast contains only a single KPNA (Srp1) (Oka and Yoneda, 2018). On the basis of homology, KPNA subtypes are classified into three subfamilies: α1 (KPNA1, KPNA5 and KPNA6), α2 (KPNA2 and KPNA7) and α3 (KPNA3 and KPNA4) (Pumroy and Gingolani, 2015; Smith et al., 2018). The three subfamilies of KPNA show distinct NLS-binding specificity (Nadler et al., 1997) and exhibit multiple biological functions including cell development and differentiation, regulation of the immune response and control of tumorigenesis.

Among the identified KPNA subfamilies, KPNA4 in the α3 subfamily is involved in the innate immune response against viral infection (Li et al., 2018; Ye et al., 2017). Several KPNA4 orthologues from different species including humans (Köhler et al., 1997), mice (Mihalas et al., 2015), pigs (Chen et al., 2016) and chickens (Caldwell et al., 2005) have been identified. In mammals, human KPNA4, a specific adaptor molecule, mediates the nuclear import of interferon (IFN) transcriptional regulators, such as NF-κB p50/p65 heterodimers and IFN regulatory factor (IRF) 3, thereby regulating type 1 IFN-mediated immune responses (Canton et al., 2018; Shaw et al., 2005). Recently, pig KPNA4 has been demonstrated to bind pig IRF3 and initiate IFN-β production during porcine circovirus infection (Li et al., 2018). However, the role of avian KPNA4, especially duck KPNA4 (duKPNA4), in type 1 IFN expression was unknown. The IRF3 gene is absent in ducks, and the functions of duck IRF7 (duIRF7) complement most of IRF3’s functions in regulating type 1 IFN expression (Magor et al., 2013). Therefore, we sought to explore whether duKPNA4 interacts with duIRF7 and induces type 1 IFN response.
Fig. 1. Sequence analysis of duKPNA4. (A) Sequence alignment of duKPNA4 with its orthologues. The amino acid sequence of duKPNA4 is compared with those of chicken (NM_001007963.1), helmeted guineafowl (XM_021393958.1), human (NM_002268.5) and mouse (NM_008467.4) KPNA4. IBB, N-terminal importin β-binding domain. ARM, armadillo repeats. (B) A phylogenetic tree of KPNA4 was constructed by neighbor-joining by using amino acid sequences aligned in MEGA; the scale bar is 0.02. The duKPNA4 cloned in this study is labeled with a red dot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Domestic duck farming is a popular business in China. In addition, ducks, as an amplification host, are susceptible to Japanese encephalitis virus (JEV) infection (Xiao et al., 2018). JEV is a single-stranded positive-sense RNA virus of the flavivirus genus that is transmitted by mosquitoes and vertebrate-amplifying hosts including birds (Dhanda et al., 1977). JEV infection causes stunted growth and death in ducklings (Xiao et al., 2018). In addition, JEV NS5 protein interacts with human KPNA4 and inhibits the nuclear translocation of IRF3 and NF-κB in human cells (Ye et al., 2017). We therefore cloned the duKPNA4 gene and analyzed its role in type I IFN expression as well as the antiviral response against JEV in primary duck embryo fibroblasts (DEFs).

2. Materials and methods

2.1. Experimental animals, cells and viruses

Specific pathogen free (SPF) ducks, clinically healthy ducks and SPF duck embryos were purchased from the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, China. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Veterinary Research Institute (IACUC No: Shvri-po-2016060501) and were performed in compliance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People’s Republic of China, Policy No. 2006 398). DEFs were prepared from 11- to 13-day-old SPF duck embryos and were grown in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco). The JEV SH15 strain (NCBI accession No. MH753130) was maintained in our laboratory.

2.2. duKPNA4 gene cloning, sequence alignment and homology analysis

The open reading frame (ORF) encoding full-length duKPNA4 was amplified by polymerase chain reaction (PCR) from the spleens of SPF ducks. The forward primer duKPNA4-F and reverse primer duKPNA4-R (Supplementary Table 1) for amplification of duKPNA4 were designed according to the predicted sequence of duKPNA4 (NCBI accession XM_027464724.1). The amplified sequence was confirmed by DNA sequencing and cloned into the pCDNA3.0 vector to express duKPNA4 tagged with HA (duKPNA4-HA). The multiple sequence alignment of duKPNA4 with chicken (NM_001007963.1), helmeted guineafowl
KPNA4 was performed with Geneious software. The functional domains of duKPNA4 were analyzed with the SMART website (http://smart.embl-heidelberg.de/). The phylogenetic tree of KPNA4 was constructed by neighbor-joining in MEGA version 6.06.

2.3. Analysis of duKPNA4 expression in different tissues

Samples of issues including the spleen, liver, ileum, jejunum, proventriculus, lung, kidney, gizzard, duodenum, brain, bursa, heart, appendix and blood were collected from three 10-day-old SPF ducks and three 10-day-old clinically healthy ducks. Total RNAs from different tissues were extracted with TRIzol reagent according to the manufacturer’s instructions (Takara, Dalian, China) and subsequently reverse transcribed to cDNA with a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). The relative expression levels of duKPNA4 in different tissues were determined by quantitative real-time RT-PCR (qRT-PCR) with TB Green™ Premix Ex Taq™ II (Takara) and primers qduKPNA4-F and qduKPNA4-R (Supplementary Table 1). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control.

2.4. Detection of the subcellular localization of duKPNA4 in DEFs

DEFs were transfected with plasmid for expression of duKPNA4-HA or empty vector in the indicated amounts (250, 500 and 1000 ng) for 24 h and stimulated with poly(I:C) treatment (+poly(I:C)) or left untreated (-poly(I:C)) for an additional 12 h. The expression of duKPNA4-HA in the transfectants was confirmed by western blotting with anti-HA antibody. The expression of IFN-α and IFN-β at the mRNA level was detected by qRT-PCR and normalized to the expression of the GAPDH gene. The concentrations of IFN-α and IFN-β proteins in the supernatants were detected by ELISA. Results are presented as the mean ± standard error from three independent experiments. **, P < 0.01; *, P < 0.05 tested by Student’s t-test.
at a concentration of 1 \( \mu \text{g/mL} \) for 12 h. The DEFs were fixed in 4% paraformaldehyde for 1 h, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin for 1 h. Subsequently, the cells were incubated with mouse anti-HA antibody (Sigma, St Louis, MO, USA) for 1 h and treated with donkey anti-mouse IgG(H + L) antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 h. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Fluorescence images were taken with a fluorescence microscope (Carl Zeiss, Zena, Germany).

### 2.5. Effect of duKPNA4 on type I IFN expression

DEFs at 70–90% confluence were transfected with plasmids for expression of duKPNA4-HA or duIRF7-Myc for 24 h and stimulated with poly(I:C) treatment (+poly(I:C)) or without poly(I:C) treatment (-poly(I:C)) for an additional 12 h. (A) The expression of duKPNA4-HA and duIRF7-Myc was detected by IFAs with anti-HA antibody and anti-Myc antibody, respectively. Nuclei were stained with DAPI. (B) The transfectants stimulated with poly(I:C) treatment were subjected to co-immunoprecipitation assays with anti-HA antibody for immunoprecipitation and anti-Myc antibody for detection of immunoprecipitated duKPNA4-HA. (C and D) The transfectants stimulated with poly(I:C) treatment were harvested for isolation of the cytoplasmic and nuclear extracts. The protein levels of duKPNA4-HA and duIRF7-Myc in the nuclear extracts were detected by western blotting. Lamin B1 was detected as the nuclear marker (C). Intensities of protein bands were determined by densitometric analysis. Relative protein levels of duKPNA4-HA and duIRF7-Myc in the nuclear extracts were normalized to those in the WCL and plotted (D). The significant difference was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

\[ **, P < 0.01; *, P < 0.05. \]

![Fig. 5. Interaction between duKPNA4 and duIRF7.](image)

2.6. Co-immunoprecipitation and western blot analysis

The ORF of duIRF7 was amplified from duck spleen by RT-PCR according to the sequence of duIRF7 (GenBank accession No. MG707077.1). The amplified sequence was confirmed by DNA sequencing and cloned into the pCDNA3.0 vector to express duIRF7 tagged with Myc (duIRF7-Myc) for co-immunoprecipitation assays. DEFs were co-transfected with recombinant plasmids for expression of duKPNA4-HA and duIRF7-Myc and incubated for 24 h. The transfectants were stimulated with poly(I:C) (1 \( \mu \text{g/mL} \)) for 12 h and lysed in ice-cold RIPA lysis buffer (Millipore, Billerica, MA, USA) containing 1 mM phenylmethyl sulfonyl fluoride for 30 min at 4°C. The supernatants of cell lysates were collected and incubated with anti-HA Protein-G-Sepharose beads for 4 h at 4°C. The beads were washed three times with phosphate buffered saline with 0.05% Tween-20, resuspended in 1× SDS-PAGE sample buffer and subjected to SDS-PAGE. The immunoprecipitated protein complexes were detected with western blot analysis with anti-Myc antibody (Millipore) and mouse anti-HA antibody (Sigma), as described previously (Zhao et al., 2019).
2.7. Analysis of the effects of duKPNA4 on the nuclear translocation of duIRF7

DEFs were co-transfected with recombinant plasmids for expression of duKPNA4-HA and duIRF7-Myc and incubated for 12 h. The transfectants were stimulated with poly(I:C) (1 μg/mL) for 12 h and lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Invitrogen) to obtain cytoplasmic and nuclear extracts. The protein levels of duIRF7-Myc in the nuclear extract were determined by western blot analysis. Lamin B1 was detected as a nuclear marker with mouse anti-LaminB1 antibody (Cell Signaling, Danvers, MA, USA).

2.8. Analysis of the effects of duKPNA4 on JEV replication

DEFs were transfected with plasmid for expression of duKPNA4-HA and incubated at 37 °C for 24 h. The transfectants were infected with the JEV SH15 strain at a multiplicity of infection (MOI) of 0.5 and incubated at 37 °C for the indicated times. The JEV-infected cells were collected at 24, 36- and 48-h post-infection (hpi) for analysis of JEV replication. The JEV titers in the supernatants were determined with 50% tissue culture infective dose (TCID50) assays. The protein levels of JEV NS3 in the cells were detected by western blotting with polyclonal antibody specific to JEV NS3 (Deng et al., 2016). The β-actin gene was used as the internal control. The primers used are listed in Supplementary Table 1.

3. Results

3.1. Sequence analysis of duKPNA4

The ORF of the duKPNA4 gene was cloned from duck spleen, which was 1563 bp in length and encoded a 520-amino acid protein (Fig. 1A). The nucleotide sequence of duKPNA4 has been deposited in GenBank under accession number MN175601. Analysis of the deduced amino acid sequence revealed that duKPNA4 had 98.7%, 98.7%, 98.1% and 97.3% sequence similarity to the chicken, helmeted guineafowl, human and mouse KPNA4 sequences, respectively (Fig. 1A), and showed a high degree of sequence similarity to orthologues in other species. Structurally, duKPNA4 contained three domains that were conserved in all orthologues of other species, including an N-terminal importin β-binding (IBB) domain, a series of armadillo (ARM) repeats and a conserved short acidic cluster (Fig. 1A). The ARM repeats are essential for NLS-binding and are composed of eight regions (ARM1, 2, 3, 4, 5, 6, 7 and 8), which shared nearly 100% sequence identity with sequences from other species (Fig. 1A). Phylogenetic analysis indicated that duKPNA4 clustered together with chicken and helmeted guinea-fowl KPNA4 in the bird group and was closest to chicken KPNA4 (Fig. 1B).

3.2. Tissue distribution of duKPNA4

To examine the tissue distribution of duKPNA4 expression, we collected different tissues from clinically healthy ducks and SPF ducks and examined duKPNA4 mRNA expression by qRT-PCR. duKPNA4 was ubiquitously expressed at varying levels in various tissues, including the...
spleen, liver, ileum, jejunum, proventriculus, lung, kidney, gizzard, duodenum, brain, bursa, heart, appendix and blood; the expression profiles were similar between clinically healthy ducks (Fig. 2A) and SPF ducks (Fig. 2B). Among the examined tissues, the blood showed the highest level of expression in both SPF and clinically healthy ducks, followed by the kidney, lung, gizzard, spleen, and duodenum, whereas low levels of expression were observed in the ileum and jejunum (Fig. 2).

3.3. Nuclear translocation of duKPNA4 in response to poly(I:C) stimulation

The subcellular location of duKPNA4 was examined by IFA. Because of a lack of a commercial antibody specific to duKPNA4, we exogenously expressed duKPNA4-HA in DEFs and detected the subcellular location of the expressed duKPNA4 with anti-HA antibody. The duKPNA4-HA was distributed in both the cytoplasm and nucleus, with predominant expression in the cytoplasm (Fig. 3A). However, in response to poly(I:C) stimulation, duKPNA4-HA translocated from the cytoplasm to the nucleus, showing a predominant accumulation in the nucleus (Fig. 3A–B). These data indicated that poly(I:C) stimulation promoted the translocation of duKPNA4 from the cytoplasm to the nucleus, thus implying a possible role of duKPNA4 in type I IFN expression in ducks.

3.4. duKPNA4 is involved in type I IFN expression

To analyze whether duKPNA4 might be involved in type I IFN expression, we transfected DEFs with plasmid for expression of duKPNA4-HA at different doses in the presence and absence of poly(I:C) stimulation, and detected the expression of interferon-α (IFN-α) and interferon-β (IFN-β) in the cells by qRT-PCR and ELISA. The expression of duKPNA4-HA was confirmed by western blotting with anti-HA antibody (Fig. 4A). Overexpression of duKPNA4-HA significantly promoted the expression of IFN-α and IFN-β at the mRNA level in a dose-dependent manner in the presence of poly(I:C) stimulation, as compared with the expression in the control vector groups (Fig. 4B). The promotion of IFN-α and IFN-β expression by duKPNA4-HA was further confirmed at the protein level. The concentrations of IFN-α and IFN-β protein in the supernatants of duKPNA4-HA transfected cells were clearly higher than those in the control vector groups (Fig. 4C).

To further confirm the involvement of duKPNA4 in type I IFN expression, we knocked down the expression of endogenous duKPNA4 in DEFs by RNA interference with siRNAs (siduKPNA4-1, siduKPNA4-2 and siduKPNA4-3) targeting three different regions of duKPNA4 mRNA (Supplementary Table 2) and analyzed the effects on the expression of IFN-α and IFN-β in the cells by qRT-PCR and ELISA. The expression of duKPNA4 mRNA in the siRNA-treated cells was confirmed at the mRNA level by qRT-PCR (Fig. 4D), because of a lack of a commercial antibody specific to duKPNA4. In response to poly(I:C) stimulation, the expression of IFN-α and IFN-β was significantly elevated at both the mRNA and protein levels in the cells without poly(I:C) stimulation. However, the elevated expression of IFN-α and IFN-β was significantly reduced at both the mRNA and protein levels in cells treated with siRNAs (siduKPNA4-1, siduKPNA4-2, and

Fig. 7. Effect of duKPNA4 on JEV replication. DEFs were transfected with plasmid for expression of duKPNA4-HA or empty vector for 24 h and subsequently infected with JEV at 0.5 MOI. The JEV-infected cells were collected at 24, 36 and 48 hpi for monitoring JEV replication. (A) JEV titers in the supernatants were detected with TCID_{50} assays. (B) The relative number of RNA copies of JEV E in the cells was measured by qRT-PCR and normalized to the expression of the β-actin gene. (C) The protein levels of JEV NS3 in the cells were detected by western blotting with anti-JEV NS3 antibody. (D) The intensities of protein bands were determined by densitometric analysis. Relative protein levels of JEV NS3 were normalized to those of β-actin and are presented relative to the level in duKPNA4-HA transfected cells collected at 24 h post infection (set as 1). Results are presented as the mean ± standard error from three independent experiments. **, * P < 0.01; *, P < 0.05 tested by Student’s t-test.
siduKPNA4-HA) (Fig. 4E–F), thus suggesting that the silencing of endogenous duKPNA4 expression eliminated the production of IFN-α and IFN-β in DEFs after poly(I:C) stimulation. Together, these data indicated that duKPNA4 is involved in type I IFN expression.

3.5. duKPNA4 interacts with duIRF7 and promotes its nuclear translocation

IRF3 and IRF7 play critical roles in type I IFN-mediated innate immunity (Takeuchi and Akira, 2009). In mammalian cells, KPNA4 interacts with IRF3 and subsequently promotes its translocation from the cytoplasm to the nucleus, thus triggering the expression of type I IFN (Li et al., 2018). The IRF3 gene is absent in the duck genome; therefore duIRF7 substitutes for most of IRF3's functions in regulating type I IFN expression (Chen et al., 2018). Given that duKPNA4 upregulated type I IFN expression, we investigated whether duKPNA4 might interact with duIRF7 and promote the translocation of duIRF7 from the cytoplasm to the nucleus. DEFs were co-transfected with plasmids for expression of duKPNA4-HA and duIRF7-Myc, and their co-localization was determined by IFAs in the presence or absence of poly(I:C) stimulation (Fig. 5A). However, in response to poly(I:C) stimulation, both the expressed duKPNA4-HA and duIRF7-Myc translocated from the cytoplasm to the nucleus, thereby upregulating type I IFN expression (Fig. 5C). The expressed duKPNA4-HA and duIRF7-Myc mainly co-localized in the cytoplasm in the absence of poly(I:C) stimulation (Fig. 5A). However, in response to poly(I:C) stimulation, both the expressed duKPNA4-HA and duIRF7-Myc translocated from the cytoplasm to the nucleus and co-localized in the nucleus (Fig. 5A), thus suggesting a possible interaction between duKPNA4 and duIRF7. To confirm this interaction, we performed co-immunoprecipitation in DEFs co-transfected with plasmids for expression of duKPNA4-HA and duIRF7-Myc, by using anti-HA antibody for immunoprecipitation and anti-Myc antibody for detection of the immunoprecipitated proteins. The duKPNA4-HA co-immunoprecipitated with duIRF7-Myc (Fig. 5B), thus revealing that duKPNA4 interacted with duIRF7 in DEFs. To further analyze whether duKPNA4 promoted the nuclear translocation of duIRF7, we extracted the nuclear fractions from DEFs co-transfected with plasmids for expression of duKPNA4-HA and duIRF7-Myc, in the presence of poly(I:C) stimulation, and performed western blotting analysis to detect the protein levels of duKPNA4-HA and duIRF7-Myc. The expression of duKPNA4-HA and duIRF7-Myc in the whole cell lysates (WCL) of DEFs was confirmed by western blotting (Fig. 5C). Analysis of changes in the protein levels of duIRF7-Myc in the nuclear fractions revealed that the nuclear accumulation of duIRF7-Myc increased, an effect correlated with the increased nuclear accumulation of duKPNA4-HA (Fig. 5C and D), thus indicating that duKPNA4 facilitated the nuclear translocation of duIRF7, thereby upregulating type I IFN expression.

3.6. duKPNA4 facilitates type I IFN expression during JEV infection

Given that JEV infection induces type I IFN expression (Zhang et al., 2018) and that duKPNA4 was involved in type I IFN expression in the presence of poly(I:C) stimulation (Fig. 4), we investigated the involvement of duKPNA4 in type I IFN expression during viral infection by using JEV as a model. DEFs were infected with JEV at different MOI (0.5, 1 and 5), and total RNA was extracted from JEV-infected cells to detect the endogenous mRNA expression levels of duKPNA4. The qRT-PCR analysis indicated that JEV infection did not significantly affect the mRNA expression of endogenous duKPNA4 in DEFs (Fig. 6A). However, the subcellular location of duKPNA4-HA shifted from the cytoplasm to the nucleus in DEFs in response to JEV infection (Fig. 6B), similarly to the translocation induced by poly(I:C) stimulation (Fig. 3). Analysis of type I IFN expression in JEV-infected DEFs with or without overexpression of duKPNA4-HA revealed that exogenous expression of duKPNA4-HA significantly elevated the mRNA levels of IFN-α and IFN-β induced by JEV infection, as compared with the levels in JEV-infected DEFs without duKPNA4-HA expression (vector control) (Fig. 6C). The expression of duKPNA4-HA in JEV-infected DEFs was confirmed by western blotting (Fig. 6D). Together, these data indicated that duKPNA4 resulted in nuclear translocation of duIRF7 and facilitated type I IFN expression during JEV infection, thereby implying its involvement in the type I IFN-mediated antiviral response.

3.7. duKPNA4 inhibits JEV replication in DEFs

On the basis of the observation that duKPNA4 facilitated type I IFN expression during JEV infection (Fig. 6), we investigated the effect of duKPNA4 on JEV replication. DEFs were transfected with plasmid expressing duKPNA4-HA or empty vector and subsequently infected with JEV. JEV replication was monitored by TCID₅₀ assays and qRT-PCR. As shown in Fig. 7A, the JEV titers in DEFs transfected with plasmid expressing duKPNA4-HA were significantly lower than those in DEFs transfected with empty vector at 24, 36 and 48 hpi. The relative number of RNA copies of JEV E (Fig. 7B) and the protein levels of JEV NS3 (Fig. 7C and D) in DEFs transfected with plasmid for expression of duKPNA4-HA were also significantly lower than those in DEFs transfected with empty vector, thus confirming the inhibitory effect of duKPNA4-HA on JEV replication.

4. Discussion

Mammalian KPNA4 plays a role in regulating the nuclear transport of type I IFN transcription factors, thereby triggering the expression of type I IFN and the subsequent immune response against virus infection (Zhu et al., 2015; Chen et al., 2016; Li et al., 2018). However, the role of avian KPNA4 in type I IFN expression was unknown. The current study aimed to clone duck KPNA4 and analyze its role in type I IFN expression as well as the antiviral response.

The cloned duKPNA4 comprised 520 amino acid and shared 97.3–98.7% sequence similarity with its orthologues from chicken, helmeted guineafowl, human and mouse, thus suggesting that KPNA4 has been highly conserved through evolutionary history (Goldfarb et al., 2004; Miyamoto et al., 2016). The IBB domain involved in binding importin β (Kobe, 1999), the ARM repeats functioning as internal cargo-NLS-binding sites mediating the nuclear translocation of cargo protein (Miyamoto et al., 2016), and the short acidic cluster that acts as a binding region for the nuclear exporter of importin α (Goldfarb et al., 2004) were highly conserved in duKPNA4. These observations suggested that duKPNA4 might have biological roles similar to those of its orthologues from mammals. Indeed, in response to poly(I:C) stimulation, duKPNA4 facilitated the expression of type I IFN, showing a conserved role in type I IFN expression similar to that of human, mouse and pig KPNA4 (Li et al., 2018; Xinghui et al., 2012).

Mechanistically, mammalian KPNA4 acts as a specific adaptor molecule that mediates the nuclear translocation of NF-κB p50/p65 heterodimers and IRF3, thereby inducing type I IFN expression (Fagerlund et al., 2005; Li et al., 2018). Knockdown of human KPNA4 in MCF-7 cells significantly inhibits NF-κB activity and impairs IFN-α/β expression, thus leading to immunological dysfunction (Zhu et al., 2015), whereas up-regulation of KPNA4 enhances NF-κB activation (Chen et al., 2016). Porcine KPNA4 has been found to be involved in the IRF3-mediated type I IFN signaling pathway during porcine circovirus type 2 infection (Li et al., 2018). In ducks, the toll-like receptor signaling pathway and the retinoic-acid-inducible gene I signaling pathway largely resemble the human pathways (Evseev and Magor, 2019) and play essential roles in the regulation of type I IFN expression and host antiviral response (Yu and Levine, 2011). Notably, IRF3, as a downstream molecule in the toll-like receptor signaling pathway and the retinoic-acid-inducible gene I signaling pathway, is absent in ducks, and duIRF7 instead performs most functions of IRF3 in inducing type I IFN expression (Santhakumar et al., 2017). In the current study, duKPNA4 interacted with duIRF7 and facilitated the nuclear translocation of duIRF7. Mechanistically, duKPNA4 promotes type I IFN expression, probably via the duIRF7-mediated type I IFN signaling pathway, in contrast to mammalian KPNA4.
Human KPNA4 is detected in nearly all tissues at both the mRNA and protein levels (Nachury et al., 1998). In this study, we detected duKPNA4 expression only at the mRNA level because of a lack of a commercial antibody specific to duKPNA4. The expression of duKPNA4 was detectable in all tissues tested, showing a ubiquitous expression pattern similar to that of human KPNA4 (Goldfarb et al., 2004; Miyamoto et al., 2016), thus suggesting the multiple biological functions of duKPNA4, such as involvement in antiviral immune response. Indeed, in response to JEV infection, duKPNA4 translocated from the cytoplasm to the nucleus and elevated the type I IFN expression induced by JEV infection. Furthermore, exogenous expression of duKPNA4 significantly inhibited JEV replication in DEFs, thereby suggesting the involvement of duKPNA4 in the host immune response against viral infection in ducks. This observation was consistent with a previous observation in humans, in which human KPNA4 has been found to have a role in the inhibition of JEV replication (Ye et al., 2017).

Given the highly conserved amino acid sequence similarity and the conserved role in type I IFN expression, KPNA4 may be an important molecule in type I IFN signaling as well as type I IFN-mediated antiviral response, and may be targeted by viruses to antagonize the type I IFN-mediated antiviral response. Indeed, the 4b protein of Middle East respiratory syndrome coronavirus binds KPNA4 and impairs the interaction between KPNA4 and the NF-κB-p65 subunit, thus interfering with the host innate immune response (Canton et al., 2018). JEV NS5 protein competitively binds KPNA4 and inhibits the nuclear translocation of IFN transcription factors, thus suppressing IFN expression (Ye et al., 2017). These previous observations together with our results emphasize the importance of KPNA4 in the type I IFN-mediated antiviral response. However, these roles must be further mechanistically explored to gain more insight into the full biological functions of KPNA4.

In summary, we cloned the duKPNA4 gene, which encoded a 520 amino acid protein with 97.3–98.7% sequence similarity to its orthologues from other species. duKPNA4 was extensively expressed in various tissues in ducks and was found to be involved in type I IFN expression in response to poly(I:C) stimulation. duKPNA4 was found to interact with duSRF7 and promote the nuclear translocation of duSRF7, thereby triggering type I IFN expression. In response to JEV infection, duKPNA4 translocated from the cytoplasm into the nucleus and elevated the expression of type I IFN expression. Exogenous expression of duKPNA4 significantly inhibited JEV replication. Together, these data suggested that duKPNA4 is involved in type I IFN expression as well as the type I IFN-mediated antiviral response.

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Appendix A. Supplementary data

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In summary, we cloned the duKPNA4 gene, which encoded a 520 amino acid protein with 97.3–98.7% sequence similarity to its orthologues from other species. duKPNA4 was extensively expressed in various tissues in ducks and was found to be involved in type I IFN expression in response to poly(I:C) stimulation. duKPNA4 was found to interact with duSRF7 and promote the nuclear translocation of duSRF7, thereby triggering type I IFN expression. In response to JEV infection, duKPNA4 translocated from the cytoplasm into the nucleus and elevated the expression of type I IFN expression. Exogenous expression of duKPNA4 significantly inhibited JEV replication. Together, these data suggested that duKPNA4 is involved in type I IFN expression as well as the type I IFN-mediated antiviral response.

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