Cloning, analysis, and anti–duck Tembusu virus innate immune response of Cherry Valley duck tripartite motif-containing 32

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ABSTRACT Tripartite motif-containing 32 (**TRIM32**) is an E3 ubiquitin ligase with multiple functions. In this study, we amplified TRIM32 gene from the Cherry Valley duck, and its cDNA sequence contained an open reading frame of 1,950 bp that encodes 649 amino acids. Duck TRIM32 (***duTRIM32***) mRNA was expressed in all tissues tested. A series of immune-related genes that were induced by viral infection, including interferon alfa, IL-1β, retinoic acid–inducible gene-I, Mx, and OAS, were regulated by *duTRIM32* expression. *DuTRIM32* overexpression inhibits duck Tembusu virus (**DTMUV**) replication in the early stages of viral infection. Knockdown of *duTRIM32* expression by siRNA reduced the ability of duck embryo fibroblast cells to mount a type I interferon response to DTMUV. Therefore, our results suggest that the *duTRIM32*-mediated signal pathway plays an essential role in DTMUV infection-induced innate immune response.

Key words: Cherry Valley duck, innate immunity, TRIM32, duck Tembusu virus, signal pathway

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INTRODUCTION

Innate immunity is the first line of host defense against invading microorganisms. Pattern recognition receptors recognize pathogen-associated molecular patterns and trigger signals to activate the immune response (Akira et al., 2006). The retinoic acid–inducible gene I (**RIG-I**)–like receptors detect nonself double-stranded RNA in the cytoplasm (Yoneyama et al., 2015). The RIG-I–like receptor family members RIG-I and melanoma differentiation–associated gene 5 can combine with downstream mitochondrial antiviral signaling (also known as IPS-1/VISA/Cardif) to activate downstream kinases and transcription factors (Yoneyama et al., 2015; Mills et al., 2017; Liu and Gao, 2018), which is a prerequisite for the production of type I interferon and inflammatory cytokines.

The duck Tembusu virus (**DTMUV**) is an enveloped, single-stranded positive-sense RNA virus, which is of the genus *Flavivirus*, family Flaviviridae, and Ntaya virus group (Tang et al., 2012). The DTMUV was isolated from the affected ducks during an outbreak of duck viral infection in 2010 in China. Major symptoms included severe decline of egg production in egg-laying and breeder ducks (Cao et al., 2011; Su et al., 2011). Previous studies have found that chickens, geese, and house sparrows can be infected with DTMUV (Liu et al., 2012; Tang et al., 2013a). The most remarkable thing is the potential for the DTMUV to become a zoonotic, which is a major issue for public health safety (Tang et al., 2013b). Our previous studies identified several members of pattern recognition receptors from ducks and geese, which demonstrated that they played critical roles in the host's immune response during virus infection (Wei et al., 2013; Wei et al., 2014; Jiao et al., 2015; Wei et al., 2016; Hou et al., 2020). Thus, to guide future DTMUV prevention efforts to combat this disease, it is necessary to understand more about the interaction between the virus and duck, the natural host.

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Tripartite motif (**TRIM**) is a protein family, and most of these proteins have E3 ubiquitin ligase activity, which is characterized by N-terminal really interesting new gene (**RING**) finger domain, one or more B-box-type zinc finger domain, a coiled-coil domain, and a variable C-terminal domain (Reymond et al., 2001; Nisole et al., 2005). To date, more than 75 members of the TRIM family have been identified in different species, which have various functions in cellular processes such as innate immunity, autophagy, carcinogenesis, development, differentiation, and apoptosis (Nisole et al., 2005; Hatakeyama, 2017). Tripartite motif-containing 32 (TRIM32) is an important member of the TRIM family; in mammals, it has been reported to be a crucial regulatory protein for innate immunity by targeting the stimulator of interferon genes–mediated signaling pathway during both DNA and RNA viral infection (Zhang et al., 2012). For viral RNA recognition, stimulator of interferon genes interacts with RIG-I, but not melanoma differentiation–associated gene 5, to modulate the RIG-I-mediated signaling pathway (Ishikawa and Barber, 2008). Recently, the TRIM32 gene was identified in Peking duck, and it was shown to play an important role in the type I interferon pathway, which is activated by H5N6 highly pathogenic avian influenza virus infection (Wu et al., 2020). However, the functions of duck TRIM32 (***duTRIM32***) are currently unclear.

In the present study, we cloned the TRIM32 gene from Cherry Valley duck and investigated its role in response to DTMUV infection. Our data will provide evidence that the *duTRIM32*-mediated signal pathway plays an important role in antiviral immunity.

MATERIALS AND METHODS

Animals, Cells, and Virus

A total of 10 healthy Cherry Valley ducks (1 d of age) were purchased from a farm (Tai'an, China) bred in an isolator until they were 2 wk of age. Primary duck embryonic fibroblasts (**DEF**) were isolated from 11-day-old duck embryos using mechanical dissociation followed by enzymatic digestion with 0.25% trypsin-EDTA (25200056; Gibco, Grand Island, NY) under sterile conditions. The DEF were maintained in Dulbecco's Modified Eagle Medium (11995500BT; Gibco) supplemented with 10% fetal bovine serum (A3160902; Gibco). All incubations were performed in 5% CO_2 at 37°C.

The DTMUV-FX2010 strain was used in this study, as described (Li et al., 2016a). Viral titers were determined as the median tissue culture infective dose ($TCID_{50}$)/mL by endpoint dilution of the DEF using the Reed and Muench calculation (Reed and Muench, 1938).

Expression Profiles for duTRIM32 in Cherry Valley Ducks

Three ducks (2 wk of age) were selected randomly to be euthanized, and their heart, liver, spleen, lung, kidney, brain, epencephalon, brainstem, thymus, pancreas, bursa of Fabricius, trachea, esophagus, muscular stomach, glandular stomach, skin, muscle, duodenum, jejunum, ileum, cecum, and rectum were harvested for total RNA isolation. The *duTRIM32* tissue expression was detected via quantitative real-time PCR (**qRT-PCR**).

Cloning and Bioinformatic Analysis of duTRIM32

Total RNA was extracted from the duck spleen using an RNA isolater (R401-01; Vazyme, Nanjing, China). The obtained RNA was reverse transcribed to cDNA using a HiScript II One-Step RT-PCR kit (R223-01; Vazyme). *DuTRIM32* cDNA was amplified using PCR with Rapid Taq Master Mix (P222, Vazyme), primers for the gene were designed based on the predicted sequence of *Anas platyrhynchos* TRIM32 at the National Center for Biotechnology Information. All primers used in this study were synthesized by the TSING Biological technology Company (Qingdao, China) and are shown in Table 1. The reaction conditions for PCR were as follows: 3 min 95°C for initial denaturation; 40 cycles of 95°C for 30 s and 60°C for 30 s, 72°C for 40 s; and 5 min at 72°C for the final extension. PCR was performed in a 50-μL reaction mixture. The PCR products were sent to the TSING Company for DNA sequencing. Sequences were analyzed using the basic local alignment search tool alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) tools in the National Center for Biotechnology databases. The amino acid sequence of *duTRIM32* was analyzed using the SMART (http://smart.embl.de/) program (Schultz et al., 1998). Multiple sequence alignment was performed using the ClustalX2 program (Larkin et al., 2007) and edited with the online tool BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). A phylogenetic tree was generated by the neighbor-joining method with MEGA-X (https://www.megasoftware.net/) software (Kumar et al., 2018).

Table 1. Primer information table of this research.

Primer name	Sequence (5′-3′)	Purpose
duTRIM32-F	AAATGTGAGTACCGGGCTTG	Gene cloning
duTRIM32-R	TCTCCTTCGTGCTTGTCCTT	
qduTRIM32-F	TCAAGAGGATGGGTTCCAAG	qRT-PCR
qduTRIM32-R	TGACCGAGTTGTCGTAGCTG	
qIFN-α-F	AGATGGCTCCCAGCTCTACA	qRT-PCR
qIFN-α-R	AGTGGTTGAGCTGGTTGAGG	
qIL-1β-F	TCATCTTCTACCGCCTGGAC	qRT-PCR
qIL-1β-R	GTAGGTGGCGATGTTGACCT	
qIL-2-F	GCCAAGAGCTGACCAACTTC	qRT-PCR
qIL-2-R	ATCGCCCACACTAAGAGCAT	
qMx-F	TGCTGTCCTTCATGACTTCG	qRT-PCR
qMx-R	GCTTTGCTGAGCCGATTAAC	
qOAS-F	TCTTCCTCAGCTGCTTCTCC	qRT-PCR
qOAS-R	ACTTCGATGGACTCGCTGTT	
qβ-actin-F	GGTATCGGCAGCAGTCTTA	qRT-PCR
qβ-actin-R	TTCACAGAGGCGAGTAACTT	

Abbreviations: F, forward primer; q, qRT-PCR; qRT-PCR, quantitative real-time PCR; R, reverse primer.

Construction of Expression Plasmids and Transfection

The expression plasmid pcDNA3.0-*duTRIM32*-Flag was obtained by cloning the Flag-tagged *duTRIM32* coding sequence into NotI and BamHI sites of the vector pcDNA3.0 (+) (Invitrogen, Carlsbad, CA) via homologous recombination using the Hieff Clone Plus One-Step Cloning Kit (10911; Yeasen, Shanghai, China). The plasmid construction was verified by sequencing. The DEF were incubated overnight to achieve 80–90% confluence before transfection. The expression plasmid pcDNA3.0-*duTRIM32* or the empty vector was transfected into DEF with the Lipofectamine 2000 reagent (11668; Invitrogen), as per the manufacturer's instructions.

Western Blotting

Western blotting analyses were performed to confirm pcDNA3.0-*duTRIM32*-Flag expression. After 48 h after transfection with expression plasmid or empty vector, the DEF were lysed with a RIPA lysis buffer (P0013C; Beyotime, Shanghai, China) containing 1 mM PMSF (ST506, Beyotime). Lysates were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was collected, and its protein concentration was determined. After adding 5 × SDS-PAGE sample buffer (Beyotime), samples were boiled for 10 min and run immediately on 10% SDS-PAGE gels. Protein samples were transferred to 0.22 μm PVDF membrane (Millipore) using NcmBlot Rapid Transfer Buffer (WB4600; New Cell & Molecular Biotech, suzhou, China). The PVDF membrane was blocked with NcmBlot blocking buffer (P30500; New Cell & Molecular Biotech) for 10 min at room temperature and then incubated with mouse anti-Flag monoclonal antibody (8146; Cell Signaling Technology, Shanghai, China) or anti–β-actin monoclonal antibody (3700; Cell Signaling Technology) overnight at 4°C. The membrane was then incubated with Peroxidase AffiniPure Goat Anti-Mouse IgG, the secondary antibody (33201ES60; Yeasen), for 1 h at room temperature. After each incubation, the membrane was washed thrice for 10 min each with 1x TBST. The protein bands were visualized with an NcmECL Ultra kit (P10100; New Cell & Molecular Biotech).

SiRNA Interference

The *duTRIM32* siRNA and negative control RNA were designed and synthesized by the GenePharma Company (Shanghai, China). The siRNA sequences are shown in Table 2. The DEF were seeded in to 6-well plates and *duTRIM32* siRNA or negative control siRNA was transfected into cells (2 μg/well) with the Lipofectamine 2000 reagent as per the manufacturer's instructions. After 36 h of transfection, the efficiency of the siRNA was verified by qRT-PCR.

Table 2. The sequences of pSi-RNA.

pSiRNA	Sequence (5′-3′)
pSi-NC (sense)	UUCGCGAAGGATT
pSi-NC (antisense)	ACGUGACCUGUGGAGATT
pSi-TRIM32-1 (sense)	GCUAAAGGCGGCUUCGAAATT
pSi- TRIM32-1 (antisense)	UUUCGAAGCCGCCUUUAGCTT
pSi-TRIM32-2 (sense)	CCAGGUCACGCAAGUUCUUTT
pSi-TRIM32-2 (antisense)	AAGAACUUGCGUGACCUGGTT

DuTRIM32 Antiviral Activity

To determine whether the *duTRIM32*-mediated signal pathway has antiviral ability, the overexpressing or knocked down *duTRIM32* DEF were infected with 100 $TCID_{50}$ DTMUV for 1 h and then were washed with PBS for 3 times. Cells were collected at 12, 24, 36, and 48 h post infection (**hpi**) for total RNA extraction. The RNA was used for viral RNA quantitation and cytokine detection by qRT-PCR.

Quantitative Real-Time PCR

Total RNA was prepared from tissues and DEF and reverse transcribed to cDNA using the aforementioned method. The *duTRIM32* primers (qduTRIM32-F/R) for qRT-PCR were designed using Primer3 Input (v.0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/) online software. The other primers used in this study were designed based on previously published primer sequences (Li et al., 2015; Li et al., 2016b). The qRT-PCR primers used in the experiment are shown in Table 1. QRT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Bio-Systems, Foster City, CA) using the ChamQ SYBR qPCR Master Mix (Q311; Vazyme). The reaction system was performed in 20-μL volumes. The conditions were as follows: 300 s at 95°C for predenaturing, 40 cycles of 95°C for 5 s, and 60°C for 30 s. A dissociation stage was performed to verify the specificity of the PCR products (95°C for 10s, 65°C for 10 s, 97°C for 1 s). The relative expression of each target gene was analyzed using the $2^{-\Delta\Delta Ct}$ method using duck β-actin as the internal reference (Livak and Schmittgen,

Table 3. Reference sequence information of TRIM32.

Species	GeneBank accession numbers
Gallus gallus	XP_015135179.2
Taeniopygia guttata	XP_030142727.2
Homo sapiens	AKI70278.1
Pan troglodytes	XP_024201619.1
Papio anubis	XP_021783495.1
Mus musculus	NP_001155254.1
Felis catus	NP_001295976.1
Bos taurus	NP_001069292.1
Canis lupus familiaris	XP_005627015.1
Capra hircus	XP_017908175.1
Oryctolagus cuniculus	XP_008271604.1
Panthera tigris	XP_007094393.1
Paralichthys olivaceus	XP_019937665.1
Salmo trutta	XP_029625258.1
Ovis aries	XP_027819877.1
Anas platyrhynchos	XP_032056135.1

Abbreviation: TRIM32, tripartite motif-containing 32.

2001). At least 3 independent experiments were performed for each sample.

Statistical Analysis

All experiments were conducted at least 3 times. All experimental data were presented as mean ± SEM. A Student t test was used to determine the statistical significance of the differences using GraphPad Prism 8.0.1 software (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered to be statistically significant, $P < 0.01$ was highly significant, and $P < 0.001$ was extremely significant.

RESULTS

Molecular Cloning and Sequence Analysis of duTRIM32

To better understand the biological function of *duTRIM32*, we cloned and characterized TRIM32 gene

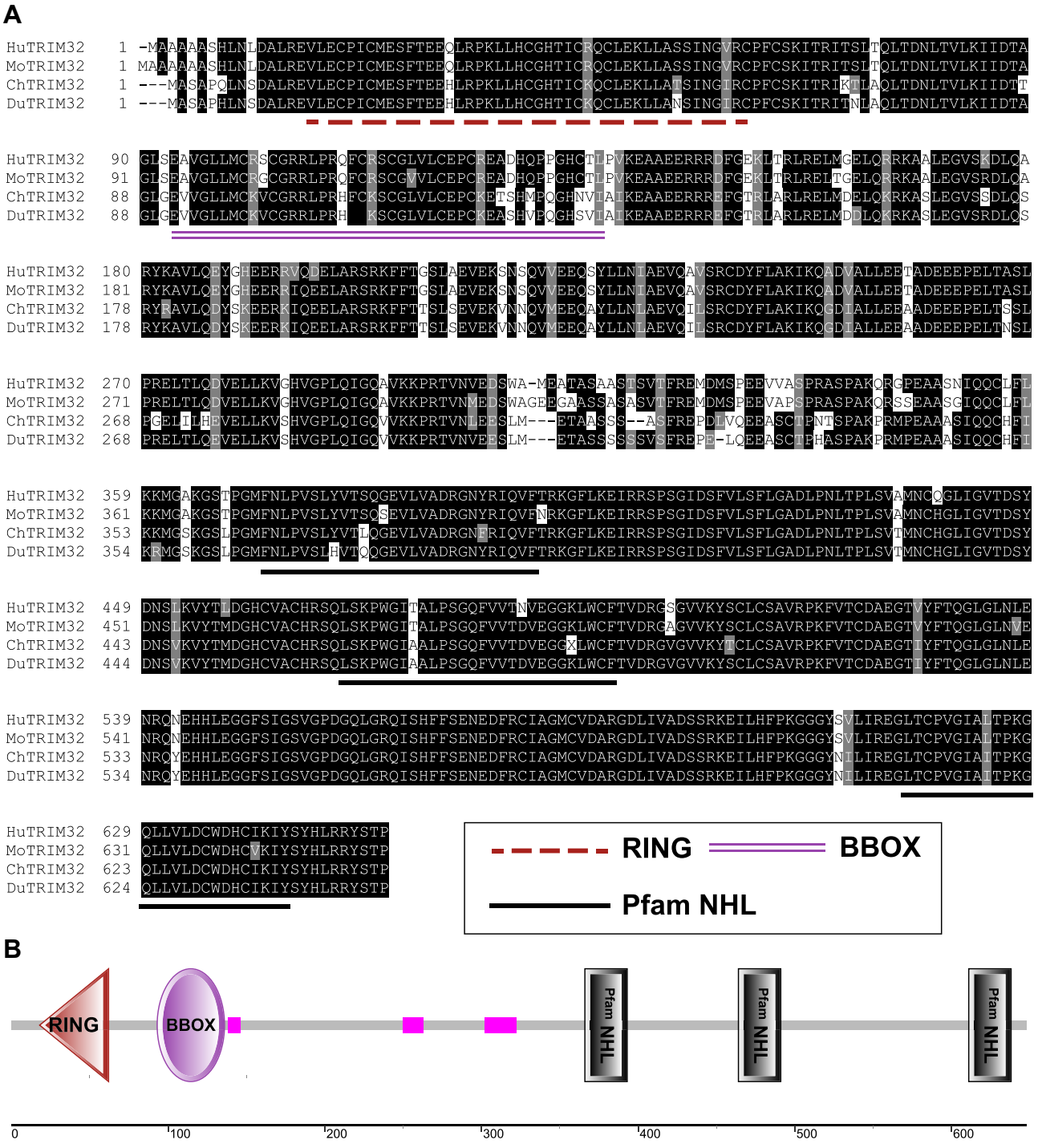
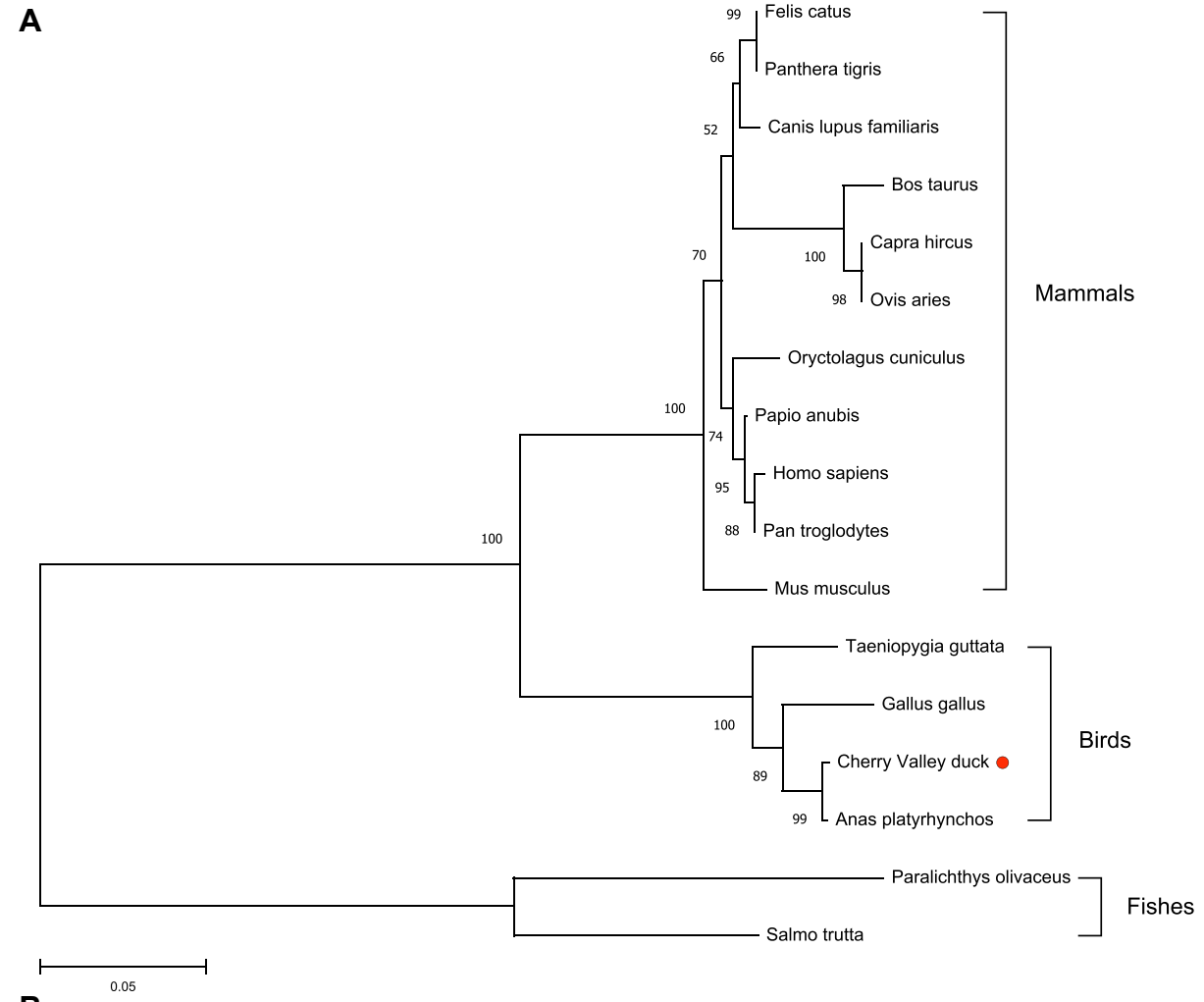


Figure 1. (A) Alignment of the deduced amino acid sequence of *duTRIM32* with other animals. Black shading indicates amino acid identity; gray shading indicates similarity (50% threshold). (B) Prediction of protein domains by the SMART program. *DuTRIM32* contains a RING domain (AA 18–62), a BBOX domain (AA 93–136), and 3 Pram NHL domains (AA 367–394, 465–492, 612–639). Abbreviations: BBOX, B-box-type; Ch, chicken; Du, Cherry Valley duck; Hu, human; Mo, mouse; RING, really interesting new gene.



B

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		93.7	83.5	83.6	83.6	83.5	83.6	81.0	83.2	81.9	83.3	83.6	61.1	62.7	81.9	95.7	96.0	1	Gallus Gallus
2	6.6		84.9	84.9	84.9	84.9	84.5	82.1	84.2	82.4	84.4	84.5	60.4	63.2	82.4	95.0	95.4	2	Taeniopygia guttata
3	18.7	17.0		99.7	99.4	96.0	97.9	93.1	97.5	93.7	97.7	97.9	61.3	64.2	93.7	84.7	84.6	3	Homo sapiens
4	18.5	17.0	0.3		99.7	96.3	98.2	93.4	97.9	94.0	98.0	98.2	61.5	64.4	94.0	84.7	84.6	4	Pan troglodytes
5	18.5	17.0	0.6	0.3		96.3	98.5	93.7	98.2	94.3	98.3	98.5	61.7	64.2	94.3	84.7	84.6	5	Papio anubis
6	18.7	17.0	4.1	3.8	3.8		96.0	92.2	95.9	92.8	95.7	96.0	61.6	64.0	92.8	84.7	84.5	6	Mus musculus
7	18.5	17.4	2.2	1.9	1.5	4.1		94.3	98.5	94.9	97.1	100.0	61.7	64.1	94.9	84.7	84.5	7	Felis catus
8	22.0	20.5	7.2	6.9	6.6	8.3	5.9		94.2	98.3	92.8	94.3	59.7	62.1	98.3	81.9	81.8	8	Bos taurus
9	19.1	17.7	2.5	2.2	1.9	4.3	1.5	6.1		94.6	97.1	98.3	61.8	64.2	94.6	84.3	83.9	9	Canis lupus familiaris
10	20.7	20.1	6.6	6.2	5.9	7.6	5.2	1.7	5.6		93.4	94.9	60.4	62.4	100.0	82.6	82.5	10	Capra hircus
11	18.9	17.6	2.3	2.0	1.7	4.4	3.0	7.6	3.0	6.9		97.1	61.3	63.9	93.4	84.6	84.5	11	Oryctolagus cuniculus
12	18.5	17.4	2.2	1.9	1.5	4.1	0.0	5.9	1.7	5.2	3.0		61.7	64.1	94.9	84.7	82.4	12	Panthera tigris
13	54.4	55.7	53.9	53.5	53.2	53.4	53.2	57.3	52.9	55.7	53.9	53.2		81.5	60.4	60.6	60.6	13	Paralichthys olivaceus
14	51.2	50.3	48.4	48.1	48.4	48.8	48.7	52.5	48.4	51.9	48.9	48.7	21.3		62.4	62.8	62.7	14	Salmo trutta
15	20.7	20.1	6.6	6.2	5.9	7.6	5.2	1.7	5.6	0.0	6.9	5.2	55.7	51.9		82.6	82.5	15	Ovis aries
16	4.5	5.1	17.1	17.1	17.1	17.1	17.1	20.7	17.7	19.9	17.3	17.1	55.2	51.1	19.9		99.7	16	Cherry Valley duck
17	4.1	4.8	17.3	17.3	17.3	17.4	17.4	20.8	18.2	20.0	17.5	20.2	55.4	51.3	20.0	0.3		17	Anas platyrhynchos
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		

Divergence

Figure 2. Phylogenetic analysis and sequence similarity of TRIM32. (A) The phylogenetic tree of the amino acid sequence of *duTRIM32* and other animals, a neighbor-joining tree was generated using MEGA-X and a 1,000 bootstrap analysis was performed. The scale bar is 0.05. GenBank accession numbers are shown in Table 3. (B) Sequence similarity analysis of TRIM32 among different species. The program was performed using MegAlign software. Abbreviation: TRIM32, tripartite motif-containing 32.

from the Cherry Valley duck (GenBank accession number: MT635446). The *duTRIM32* open reading frame contains 1,950 bp and encodes 649 amino acid residues (Figure 1A). Analysis of the functional domain of the gene using SMART software shows that the gene has a RING domain (AA 18–62), a B-box-type domain

(AA 93–136), and 3 Pram NHL domains (AA 367–394, 465–492, 612–639) (Figure 1B)

Homology analysis showed that *duTRIM32* structures are highly homologous with the predicted *Anas platyrhynchos* (mallard) TRIM32 (99.7%) and also highly similar to birds such as *Gallus gallus* (chicken) (95.7%). However, the *duTRIM32* had a lower sequence homology to fish and mammals (from 60.5 to 84.7%) (Figure 2).

DuTRIM32 is Widely Expressed in Normal Tissues

Duck *TRIM32* expression levels were detected by qRT-PCR. Duck *TRIM32* mRNA was expressed in all tissues tested. The ileum, which had the lowest *duTRIM32* expression, served as the reference tissue, and the results indicated that the *duTRIM32* gene is highly expressed in the lung, trachea, thymus, pancreas, skin, and muscle. However, *duTRIM32* expression is lower in the intestinal organs such as the jejunum, ileum, and cecum, but not in the duodenum (Figure 3). The high *duTRIM32* mRNA expression in the lung, trachea, and duodenum suggests that the gene may be involved in the host's innate immune response.

Overexpression of duTRIM32 Induces Expression of Immune-Related Genes and Inhibits DTMUV Replication In Vitro

To understand immune induction by *duTRIM32*, the expression of the plasmid was verified by Western blot using anti-Flag monoclonal antibody, with β-actin protein as the control. Cytokine mRNA levels and DTMUV replication were examined in *duTRIM32* DEF overexpression via qRT-PCR. Western blot results showed that the plasmid was successfully expressed in DEF (Figure 4A). QRT-PCR results showed that DTMUV-stimulated immune-related gene expression was upregulated by transient overexpression of *duTRIM32* in the early stage of virus infection. The interferon alfa, IL-1β, RIG-I, Mx, and OAS mRNA expression was increased 16.87 ($P < 0.01$), 3.02 ($P < 0.05$), 2.90 ($P < 0.05$), 7.13 ($P < 0.01$), and 13.23 times ($P < 0.01$) at 12 hpi, respectively (Figures 4B–4F). The proinflammatory factor IL-1β still maintained a high expression level at 24 hpi (Figures 4B and 4C). However, after 24 hpi, upregulation level of other immune-related genes except IL had decreased to some extent. The interferon alfa, RIG-I, MX, and OAS expression showed no significant difference between the *duTRIM32* overexpression group and empty vector control group at 24 hpi (Figures 4D–4F). After 36 hpi, interferon alfa, RIG-I, and MX gene expression levels were reversed (Figures 4B, 4D, and 4E). Furthermore, *duTRIM32* overexpression significantly reduced DTMUV replication efficacy, especially in the early stages of infection, and viral replication decreased by 16.83 ($P < 0.01$), 11.76 ($P < 0.01$), 17.33 ($P < 0.01$), and 2.78 times ($P < 0.01$) compared to the empty vector control group at 12, 24, 36, and 48 hpi, respectively (Figure 4G). These results suggest that *duTRIM32* promotes DTMUV-mediated expression of immune-related genes and that *duTRIM32* overexpression can inhibit viral replication in vitro.

Knockdown of duTRIM32 Reduces Expression of Immune-Related Genes and Promotes DTMUV Replication In Vitro

To investigate the role of *duTRIM32* in anti-DTMUV immune response, we transfected DEF with siRNA to

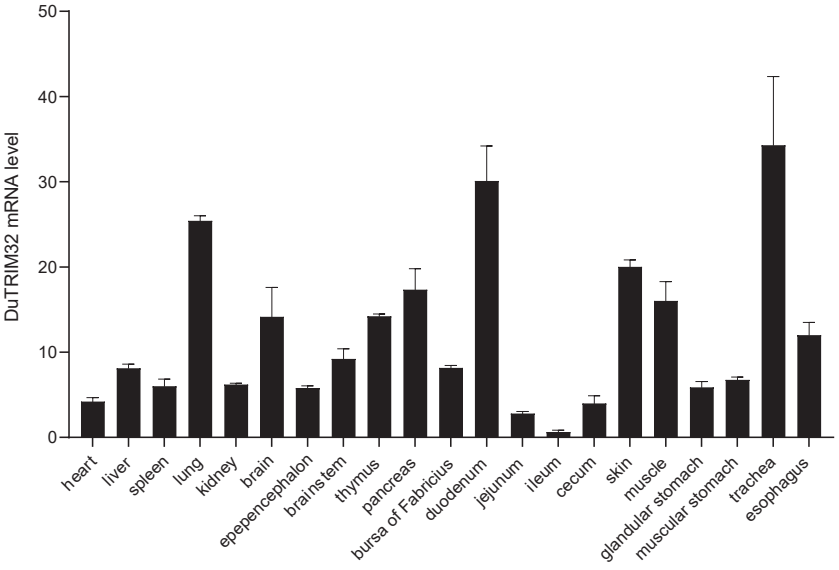


Figure 3. Tissue distribution of *duTRIM32* transcripts in healthy Cherry Valley duck. The relative mRNA levels were normalized to the expression of the β-actin gene from various tissues. Data were normalized to the spleen and error bars indicated SEM. Abbreviation: duTRIM32, duck tripartite motif-containing 32.

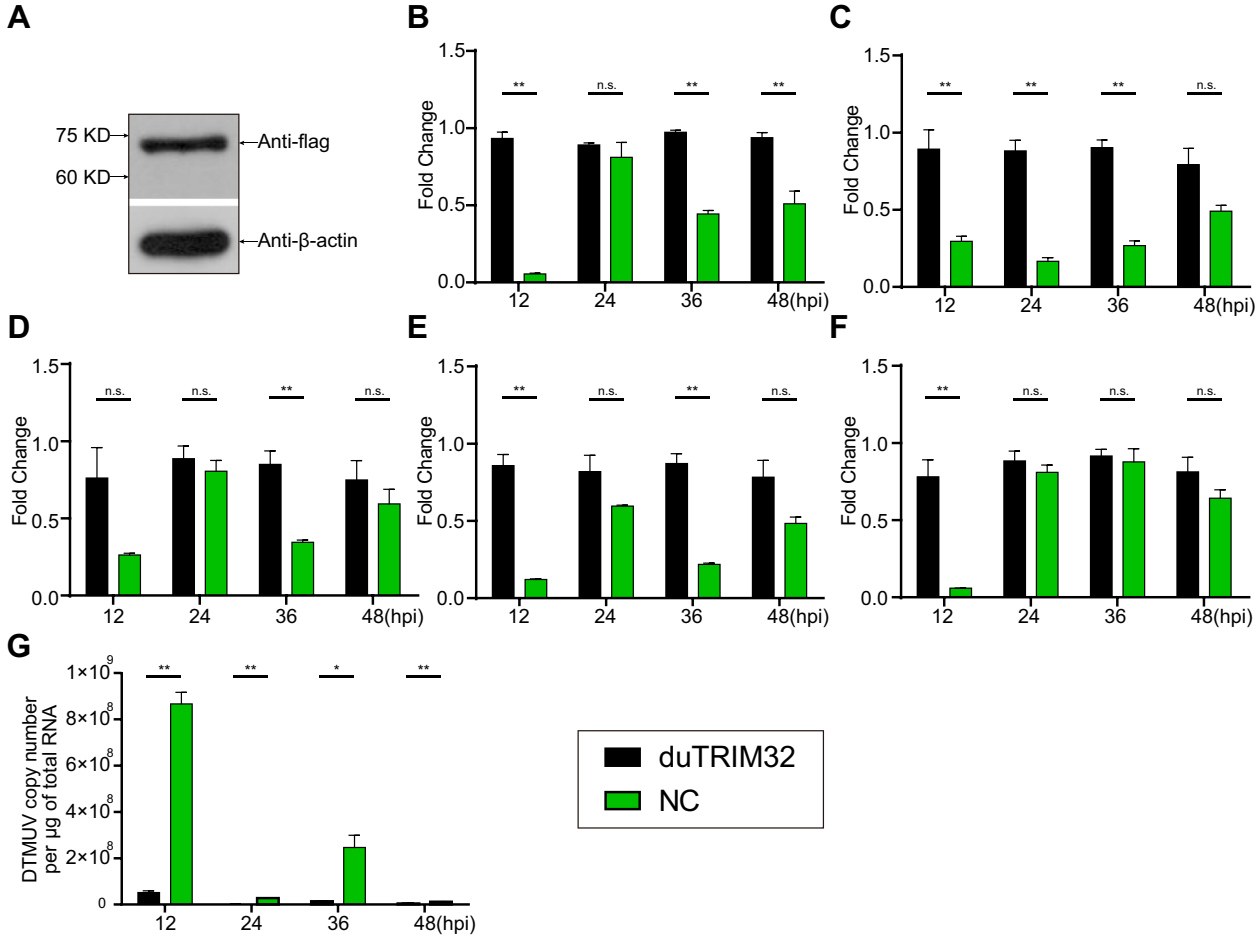


Figure 4. *DuTRIM32* overexpression promotes antiviral ability and immune-related genes production in DEF. (A) Expression of *duTRIM32* in DEF was analyzed by Western blotting using anti-Flag mAb, with β-actin protein as the control. (B–F) interferon alfa, IL-1β, RIG-I, Mx, and OAS gene expression levels were detected by qRT-PCR. (G) DTMUV copy number. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All data are expressed as means ± SEM (n = 3). Student *t* test were performed to evaluate differences. *Significant difference ($P < 0.05$); **Highly significant difference ($P < 0.01$). Abbreviations: DEF, duck embryonic fibroblasts; DTMUV, duck Tembusu virus; duTRIM32, duck tripartite motif-containing 32; n.s., no significant difference; mAb, monoclonal antibody; qRT-PCR; quantitative real-time PCR.

knock down *duTRIM32* expression. The qRT-PCR results showed that two siRNA sequences (pSi-TRIM32-1/2) were able to knock down *duTRIM32* mRNA levels, but the efficiency of pSi-TRIM32-1 was higher than that of pSi-TRIM32-2 ($P < 0.05$) (Figure 5A). Thus, pSi-TRIM32-1 was used for further experiments. At 12, 24, 36, and 48 hpi with DTMUV, virus titers and cytokine levels in the *duTRIM32* knockdown DEF were assayed by qRT-PCR. The results showed that interferon alfa, IL-1β, RIG-I, Mx, and OAS mRNA expression was reduced 46.95 ($P < 0.01$), 2.00 ($P < 0.01$), 43.53 ($P < 0.05$), 712.72 ($P < 0.01$), and 27.74 times ($P < 0.01$) at 12 hpi, respectively (Figures 5B–5F). During the entire infection period, almost all of the immune-related genes that were tested were significantly downregulated in *duTRIM32* knockdown cells that were infected with the DTMUV (Figures 5B–5F), except for RIG-I at 36 hpi (Figure 5D). In addition, knockdown of *duTRIM32* significantly increased DTMUV replication in DEF during infection, and viral replication increased 755.12 ($P < 0.01$), 24.27- ($P < 0.01$), 54.65 ($P < 0.01$), and 3.64 times at 12, 24, 36, and 48 hpi, respectively (Figure 5G). These results further illustrate that *duTRIM32* is essential for the activation of innate immune responses against DTMUV infection.

DISCUSSION

Since its discovery in China in 2010, the Tembusu virus has caused huge losses to the duck industry, and almost all duck farms in Southern China have been affected by the virus (Yan et al., 2011b). Many researchers have conducted detailed research on the disease and established a variety of clinical rapid diagnostic methods that have actively promoted timely disease diagnosis and vaccine development, and they have initiated comprehensive prevention and treatment programs to minimize the loss of economic benefits (Yan et al., 2011a; Yun et al., 2012; Chen et al., 2019). However, less work has been carried out on the pathogenic mechanism of the virus. Therefore, systematic study of the interaction between DTMUV and the host's innate immune molecules can provide a theoretical basis for further understanding the pathogenic mechanism of

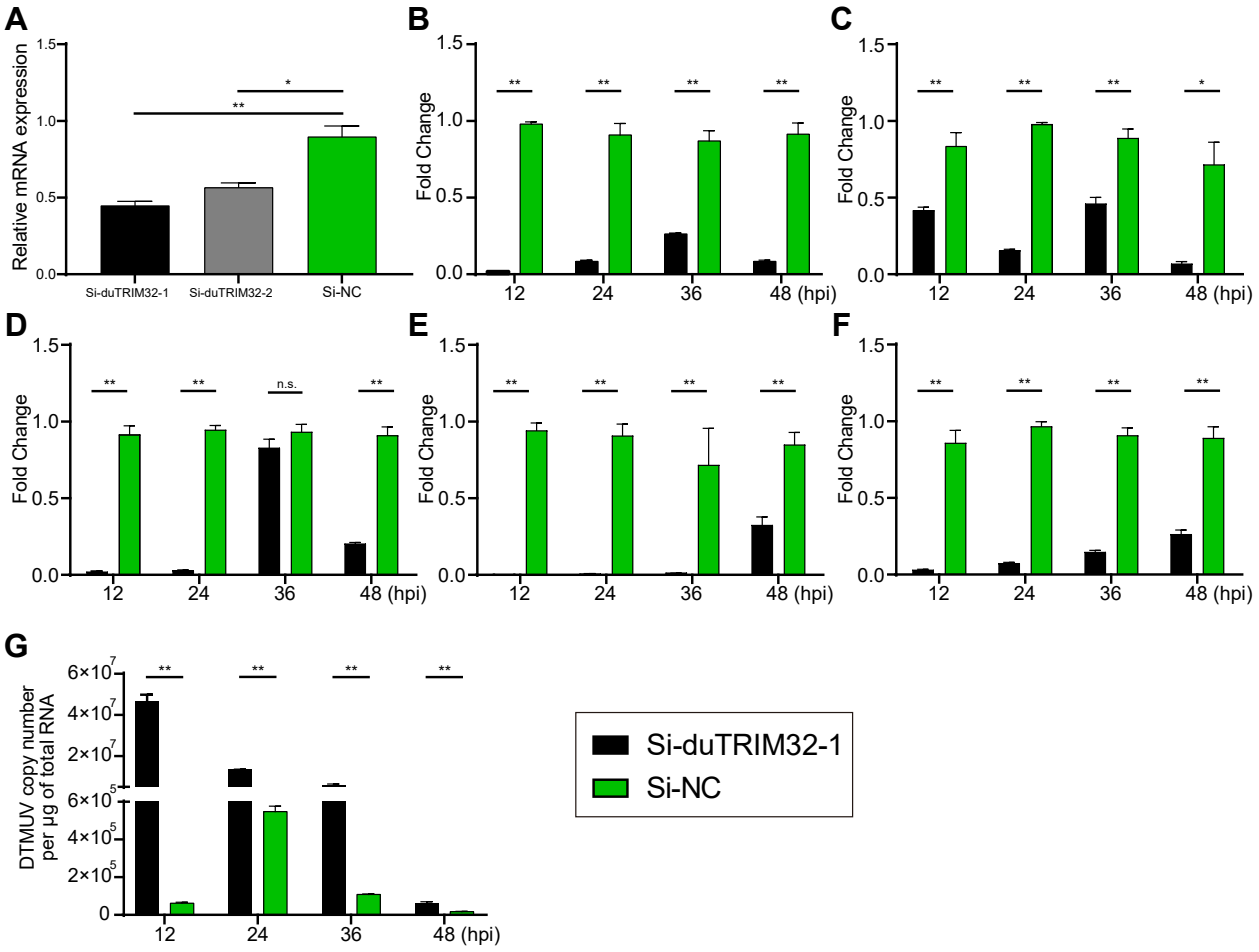


Figure 5. *DuTRIM32* knockdown decreases antiviral ability and immune-related genes production in DEF. (A) Interference efficiency of pSi-TRIM32. (B–F) interferon alfa, IL-1β, RIG-I, Mx, and OAS gene expression levels were detected by qRT-PCR. (G) DTMUV copy number. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All data are expressed as means ± SEM (n = 3). Student *t* test were performed to evaluate differences. *Significant difference ($P < 0.05$); **Highly significant difference ($P < 0.01$). Abbreviations: DEF, duck embryonic fibroblasts; DTMUV, duck Tembusu virus; duTRIM32, duck tripartite motif-containing 32; n.s., no significant difference; mAb, monoclonal antibody; qRT-PCR; quantitative real-time PCR.

the disease and developing new antiviral drugs or vaccine adjuvants.

Retinoic acid–inducible gene-I–like receptors signaling pathways play an important role in the antiviral immune response, especially for RNA viruses (Yoneyama et al., 2015). As an important indirect regulator of the RIG-I–like receptors signaling pathway, the TRIM32 gene has great potential in the future development of antiviral therapy. TRIM32 has been reported to be an important regulatory factor for innate immune system in mammals (Zhang et al., 2012). Yu et al. reported that fish TRIM32 is a critical antiviral factor during *Iridovirus* and *Nodavirus* infection (Yu et al., 2017).

In this study, the coding sequences of *duTRIM32* cDNA were cloned from Cherry Valley duck. Sequence analysis results demonstrates that the *duTRIM32* amino acid sequence shares 99.7% homology with that of the mallard, suggested that they may have almost the same functions. The *duTRIM32* coding sequences contain 1950 bp and encode 649 amino acid residues (Figure 1). Multiple sequence alignment results revealed that *duTRIM32* had an identity of 81.8–84.7% with mammals. The gene also showed similarity to chicken, with 95.7% homology.

Cherry Valley duck is one of the largest commercial duck breeds in China, but its production has long been threatened by the DTMUV (Li et al., 2016a). This work demonstrated that *duTRIM32* overexpression induced high interferon alfa and inflammatory cytokine expression that was mediated by the DTMUV (Figures 4B–4F). In the early stages of viral infection, *duTRIM32* overexpression inhibited DTMUV replication in DEF (Figure 4G). In addition, we found that knockdown of *duTRIM32* expression by specific siRNA significantly decreased expression of cytokines that were mediated by the virus (Figures 5B–5F), and virus replication was observably reduced throughout the infection period (Figure 5G). These results indicate that *duTRIM32* is involved in the production of type I interferon activation and immune-related gene mediated by DTMUV infection. This is consistent with research on TRIM32 in mammals (Zhang et al., 2012). The

expression of innate immunity-related genes is very important to resist early viral infection, and it is necessary to further activate acquired immunity (Akira et al., 2006; Kumar et al., 2011). The invasion of pathogenic microorganisms also activates the innate immune system to produce different degrees of inflammation (Takeuchi and Akira, 2010), which is a protective response by the host to ensure the removal of harmful stimuli and the process of repairing damaged tissue (Medzhitov, 2008). However, the excessive or uncontrolled release of proinflammatory cytokines (also known as cytokine storm) may cause serious damage to the host (Tisoncik et al., 2012).

In summary, we cloned, characterized phylogenetically analyzed the TRIM32 gene from the Cherry Valley duck for the first time. Duck *TRIM32* is widely expressed in normal tissues, and it plays an important role in the innate immune response against DTMUV infection. Our experimental data indicate that regulation of *duTRIM32* may be a treatment strategy for the DTMUV.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2021.101048.

REFERENCES

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. Cell 124:783–801.

Cao, Z., C. Zhang, Y. Liu, Y. Liu, W. Ye, J. Han, G. Ma, D. Zhang, F. Xu, X. Gao, Y. Tang, S. Shi, C. Wan, C. Zhang, B. He, M. Yang, X. Lu, Y. Huang, Y. Diao, X. Ma, and D. Zhang. 2011. Tembusu virus in ducks, China. Emerg. Infect Dis. 17:1873–1875.

Chen, H., M. Yan, Y. Tang, and Y. Diao. 2019. Evaluation of immunogenicity and protective efficacy of a CpG-adjuvanted DNA vaccine against Tembusu virus. Vet. Immunol. Immunopathol 218, 109953.

Hatakeyama, S. 2017. TRIM family proteins: roles in autophagy, immunity, and carcinogenesis. Trends Biochem. Sci. 42:297–311.

Hou, X., G. Liu, H. Zhang, X. Hu, X. Zhang, F. Han, H. Cui, J. Luo, R. Guo, R. Li, N. Li, and L. Wei. 2020. High-mobility group box 1 protein (HMGB1) from Cherry Valley duck mediates signaling pathways and antiviral activity. Vet. Res. 51:12.

Ishikawa, H., and G. N. Barber. 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455:674–678.

Jiao, P. R., L. M. Wei, Y. F. Song, J. Cui, S. Zhang, F. Han, R. Y. Yuan, and M. Liao. 2015. Molecular cloning and immune responsive expression of LGP2 gene, a pivotal member of the RLR gene family from Muscovy duck Cairina moschata. Poult. Sci. 94:1170–1176.

Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. Int. Rev. Immunol. 30:16–34.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. Mega X: molecular Evolutionary Genetics analysis across Computing Platforms. Mol. Biol. Evol. 35:1547–1549.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23:2947–2948.

Li, N., T. Hong, R. Li, Y. Wang, M. Guo, Z. Cao, Y. Cai, S. Liu, T. Chai, and L. Wei. 2016a. Cherry Valley ducks mitochondrial antiviral-signaling protein-mediated signaling pathway and antiviral activity research. Front Immunol. 7:377.

Li, N., T. Hong, Y. Wang, Y. Wang, K. Yu, Y. Cai, S. Liu, L. Wei, and T. Chai. 2016b. The pathogenicity of novel duck reovirus in Cherry Valley ducks. Vet. Microbiol. 192:181–185.

Li, N., Y. Wang, R. Li, J. Liu, J. Zhang, Y. Cai, S. Liu, T. Chai, and L. Wei. 2015. Immune responses of ducks infected with duck Tembusu virus. Front Microbiol. 6:425.

Liu, B., and C. Gao. 2018. Regulation of MAVS activation through post-translational modifications. Curr. Opin. Immunol. 50:75–81.

Liu, M., S. Chen, Y. Chen, C. Liu, S. Chen, X. Yin, G. Li, and Y. Zhang. 2012. Adapted tembusu-like virus in chickens and geese in China. J. Clin. Microbiol. 50:2807–2809.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408.

Medzhitov, R. 2008. Origin and physiological roles of inflammation. Nature 454:428–435.

Mills, E. L., B. Kelly, and L. A. J. O'Neill. 2017. Mitochondria are the powerhouses of immunity. Nat. Immunol. 18:488–498.

Nisole, S., J. P. Stoye, and A. Saib. 2005. TRIM family proteins: retroviral restriction and antiviral defence. Nat. Rev. Microbiol. 3:799–808.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Epidemiol. 27:493–497.

Reymond, A., G. Meroni, A. Fantozzi, G. Merla, S. Cairo, L. Luzi, D. Riganelli, E. Zanaria, S. Messali, S. Cainarca, A. Guffanti, S. Minucci, P. G. Pelicci, and A. Ballabio. 2001. The tripartite motif family identifies cell compartments. EMBO J. 20:2140–2151.

Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA 95:5857–5864.

Su, J., S. Li, X. Hu, X. Yu, Y. Wang, P. Liu, X. Lu, G. Zhang, X. Hu, D. Liu, X. Li, W. Su, H. Lu, N. S. Mok, P. Wang, M. Wang, K. Tian, and G. F. Gao. 2011. Duck egg-drop syndrome caused by BYD virus, a new Tembusu-related flavivirus. PLoS One 6:e18106.

Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. Cell 140:805–820.

Tang, Y., Y. Diao, X. Gao, C. Yu, L. Chen, and D. Zhang. 2012. Analysis of the complete genome of Tembusu virus, a flavivirus isolated from ducks in China. Transbound Emerg. Dis. 59:336–343.

Tang, Y., Y. Diao, C. Yu, X. Gao, X. Ju, C. Xue, X. Liu, P. Ge, J. Qu, and D. Zhang. 2013a. Characterization of a Tembusu virus isolated from naturally infected house sparrows (Passer domesticus) in Northern China. Transbound Emerg. Dis. 60:152–158.

Tang, Y., X. Gao, Y. Diao, Q. Feng, H. Chen, X. Liu, P. Ge, and C. Yu. 2013b. Tembusu virus in human, China. Transbound Emerg. Dis. 60:193–196.

Tisoncik, J. R., M. J. Korth, C. P. Simmons, J. Farrar, T. R. Martin, and M. G. Katze. 2012. Into the eye of the cytokine storm. Microbiol. Mol. Biol. Rev. 76:16–32.

Wei, L., J. Cui, Y. Song, S. Zhang, F. Han, R. Yuan, L. Gong, P. Jiao, and M. Liao. 2014. Duck MDA5 functions in innate immunity against H5N1 highly pathogenic avian influenza virus infections. Vet. Res. 45:66.

Wei, L., P. Jiao, R. Yuan, Y. Song, P. Cui, X. Guo, B. Zheng, W. Jia, W. Qi, T. Ren, and M. Liao. 2013. Goose Toll-like receptor 7 (TLR7), myeloid differentiation factor 88 (MyD88) and antiviral molecules involved in anti-H5N1 highly pathogenic avian influenza virus response. Vet. Immunol. Immunopathol. 153:99–106.

Wei, L., Y. Song, J. Cui, N. Qu, N. Wang, G. Ouyang, M. Liao, and P. Jiao. 2016. Cloning, characterization, and expression analysis of LGP2 cDNA from goose, Anser cygnoides. Poult. Sci. 95:2290–2296.

Wu, S., J. Zhang, Q. Xue, J. Liu, B. Huang, Z. He, J. Huang, S. Zu, Z. Chen, B. Zhao, M. Liao, and P. Jiao. 2020. Duck TRIM32 functions in IFN-beta signaling against the infection of H5N6 highly pathogenic avian influenza virus. Front Immunol. 11:377.

Yan, L., P. Yan, J. Zhou, Q. Teng, and Z. Li. 2011a. Establishing a TaqMan-Based Real-Time PCR Assay for the rapid detection and quantification of the newly emerged duck tembusu virus. Virol. J. 8:464.

Yan, P., Y. Zhao, X. Zhang, D. Xu, X. Dai, Q. Teng, L. Yan, J. Zhou, X. Ji, S. Zhang, G. Liu, Y. Zhou, Y. Kawaoka, G. Tong, and Z. Li. 2011b. An infectious disease of ducks caused by a newly emerged Tembusu virus strain in mainland China. Virology 417:1–8.

Yoneyama, M., K. Onomoto, M. Jogi, T. Akaboshi, and T. Fujita. 2015. Viral RNA detection by RIG-I-like receptors. Curr. Opin. Immunol. 32:48–53.

Yu, Y., X. Huang, J. Liu, J. Zhang, Y. Hu, Y. Yang, Y. Huang, and Q. Qin. 2017. Fish TRIM32 functions as a critical antiviral molecule against iridovirus and nodavirus. Fish Shellfish Immunol. 60:33–43.

Yun, T., Z. Ni, J. G. Hua, W. C. Ye, L. Chen, S. Zhang, Y. Zhang, and C. Zhang. 2012. Development of a one-step real-time RT-PCR assay using a minor-groove-binding probe for the detection of duck Tembusu virus. J. Virol. Methods 181:148–154.

Zhang, J., M. M. Hu, Y. Y. Wang, and H. B. Shu. 2012. TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. J. Biol. Chem. 287:28646–28655.