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Molecular characterization, expression, and functional analysis of chicken *TRAF6*

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ABSTRACT. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a crucial adaptor molecule of the interleukin-1 receptor/Toll-like receptor (IL-1/TLR) superfamily, which can trigger downstream signaling cascades involved in innate immunity. The function of *TRAF6* has been clarified in mammals but is poorly understood in chicken. In our study, we investigated *TRAF6* function in birds, particularly in chicken innate immune responses, by cloning and characterizing chicken *TRAF6* (*chTRAF6*). The full-length coding sequence of *chTRAF6* comprised 1638 bp and encoded a 545-amino acid protein, which shares high sequence similarity with *TRAF6* of other species and consists of four structurally conserved domains. Quantitative real-time polymerase chain reaction revealed that *chTRAF6* was widely expressed in all tested tissues and its expression was induced in chicken embryo fibroblast cells treated with poly(I:C) and poly(dA:dT). Increased expression of *chTRAF6* was observed both *in vitro* and *in vivo* following infection with Newcastle disease virus in

chickens. Taken together, these results suggest that *chTRAF6* plays a vital role in host defense against viral infection in chicken.

Key words: Chicken; *TRAF6*; Innate immune system; Infection; mRNA expression

INTRODUCTION

Tumor necrosis factor receptor-associated factor (TRAF) family members (*TRAF1-7*) are intracellular signal transducers involved in inflammation and include immune receptor superfamilies, such as the tumor necrosis factor receptor (TNFR) superfamily and interleukin-1 receptor/Toll-like receptor (IL-1/TLR) family members (Inoue et al., 2000). All TRAFs have a similar protein structure, an N-terminal region, and C-terminal TRAF domain (Rothe et al., 1994). The N-terminal contains a RING-finger domain and a series of five Zn-finger domains, which mediate protein ubiquitylation and activation of downstream signaling (Yang et al., 2015). The TRAF domain is comprised of a coiled-coil TRAF-N domain and a conserved TRAF-C domain, which is involved in self-association (Rothe et al., 1994) and interaction with various upstream regulators or downstream effectors (Zheng et al., 2010). *TRAF6* was originally isolated by yeast two-hybrid screening using CD40 as bait (Ishida et al., 1996), and independently by screening of an expressed sequence tag (EST) library (Cao et al., 1996), and has been identified as a critical element for IL-1/TLR signaling. *TRAF6* is the only TRAF family member that participates in both TNFR superfamily signaling pathways and TLR/IL-1R superfamily pathways (Chung et al., 2002).

Previous studies have shown that *TRAF6* regulates several signaling cascades involved in adaptive immunity, innate immunity, and bone metabolism (Ye et al., 2002). *TRAF6* has a unique receptor specificity that does not overlap with that of other TRAF family members (Darnay et al., 1999). *TRAF6* recognizes different binding sites of CD40 and receptor activator of nuclear factor kB (RANK), and other members of the TNFR superfamily (Pullen et al., 1998). *TRAF6* does not directly bind to members of the IL-1R/TLR superfamily (Lomaga et al., 1999). Signaling proteins interleukin-1 receptor-associated kinase 1 (IRAK1) (Cao et al., 1996), interleukin-1 receptor-associated kinase 2 (IRAK2) (Muzio et al., 1997), interleukin-1 receptor-associated kinase M (IRAK-M) (Wesche et al., 1999), and possibly interleukin-1 receptor-associated kinase 4 (IRAK4) in the IL-1R/TLR pathway interact with *TRAF6* (Suzuki et al., 2002). Members of the IRAK family are recruited to active receptors via interaction with adaptor proteins, such as myeloid differentiation factor 88 (Myd88), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), translocation associated membrane protein (TRAM), toll interacting protein (TOLLIP), and sterile alpha and HEAT/Armadillo motif containing (SARM) (Burns et al., 2000). Downstream signaling of *TRAF6* is involved in K63-linked polyubiquitination (Deng et al., 2000). In addition, the retinoic acid-inducible gene I/melanoma differentiation-associated gene 5 (RIG-I/MDA5) signaling pathway requires the mitochondrial protein ISP-1 to form a signaling complex with *TRAF6*, which leads to the activation of *TRAF6* and initiation of the innate immune response (West et al., 2011).

In recent years, the function and molecular mechanisms of *TRAF6* action in the innate immune response have been intensively studied in mammals and yet remain unknown in chicken. Multiple studies have demonstrated that *TRAF6* plays a crucial role in mammalian innate immunity (King et al., 2006). To investigate whether *chTRAF6* plays an important role

in innate immunity in chicken, we first cloned and characterized the cDNA of *chTRAF6* and predicted its structure. Then, the expression of *chTRAF6* was determined in all tissues tested. Finally, we analyzed the expression of *chTRAF6 in vivo* in chickens infected with Newcastle disease virus (NDV) and examined the expression of *chTRAF6 in vitro* in chicken embryo fibroblast cells (CEFs) stimulated with poly(I:C) or poly(dA:dT).

MATERIAL AND METHODS

Chicken, tissues, cells, and viruses

Specific pathogen-free (SPF) chickens are widely used experimental animals, and their use discounts interference by other pathogens. Forty White Leghorn SPF chickens from the house at the experiment farm of Sichuan Agricultural University (Sichuan, China) were employed for the experiment. Heart, liver, spleen, thymus, pancreas, small intestine, kidney, brain, pectoral muscle, and leg muscle were collected from three 14-day-old chickens for analysis of gene expression. All protocols used in this study were approved by Sichuan Agricultural University Animal Care and Use Committees. All tissues were snap-frozen in liquid nitrogen and stored at -80°C until further use. CEFs were prepared from 9-day-old SPF chicken embryos and maintained in DEME (Life Technologies, USA) supplemented with 10% FBS (GIBCO, Life Technologies). The cells were cultured at 37°C with 5% CO_2. NDV F48E9 and Mukteswar were obtained from the Department of Veterinary Medicine of Sichuan Agricultural University (Wenjiang, China).

chTRAF6 gene cloning and bioinformatic analysis

Primers (Table 1) were designed with Primer Premier 5.0 according to the published predicted sequence of *chTRAF6* from *Gallus gallus* (GenBank accession No. XP_004941605.1), and used to amplify *chTRAF6* cDNA by RT-PCR from spleen RNA. The amino acid sequences were blasted against the NCBI database using the BLAST program (http://blast.ncbi.nlm.nih.gov/) to determine sequence identities and deduce amino acid homology. Conserved domains of *chTRAF6* were predicted using the SMART program (http://smart.embl-heidelberg.de/). Amino acid sequences were aligned using Clustal X2 (Figure 1) and a phylogenic tree (Figure 2) was constructed by MEGA 5.1 using 13 species (Table 2).

Table 1. PCR primers used in this study.

Primer name	Primer sequence (5'- 3')	Application
TRAF6-F	AGGACCAGGTTGTCTCCTGT	Cloning
TRAF6-R	CCATCAAGTCCACAACACGG	
GAPDH-F	AGGACCAGGTTGTCTGCTGT	Real-time PCR
GAPDH-R	CCATCAAGTCGACAACACGG	
TRAF6-F	ATGGAAGCCAAGCCAGAGTT	Real-time PCR
TRAF6-R	ACAGCGCACCAGAAGGGTAT	

NDV challenge and sample collection

Thirty-six 2-week-old SPF chickens were randomly divided into three groups. Chickens in the first group were-infected intraperitoneally with NDV-F48E9, the second

group was infected with NDV Mukteswar, and the third group was treated with phosphate-buffered saline as a control under the same conditions. At 1-, 2-, and 4-days post-infection, four chickens were euthanized from each group. Immune tissues were collected from all killed chicken, including the bursa of Fabricius, spleen, and thymus, snap frozen in liquid nitrogen, and then stored at -80°C. CEFs were collected at 4-, 6-, 8-, 12-, and 16-h post-infection with 1 multiplicity of infection of NDV F48E9 or Mukteswar, leaving uninfected cells as a control.

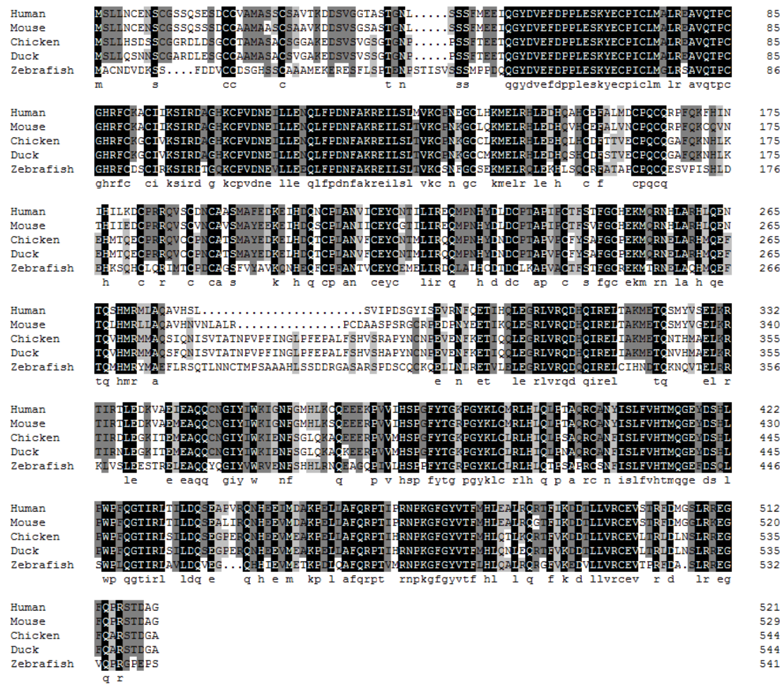

Figure 1. Alignment of human, mouse, chicken, duck, zebra fish *TRAF6* amino acid sequences, performed using CLUSTAL X2. Letters below the alignments indicate identity, and gaps are marked with dashes.

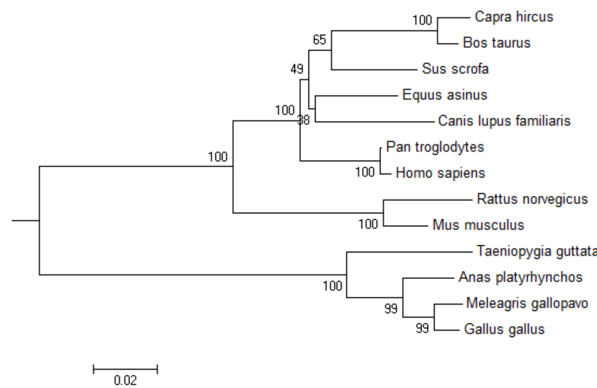

Figure 2. Phylogenetic analysis of the chTRAF6 protein. The tree was constructed using the MEGA 5.1 software and the scale bar is 0.02.

Table 2. GenBank accession No. of *TRAF6* genes used in this study.

Name of species	Accession No.
Capra hircus	XP_005690148.1
Bos taurus	NP_001192515.1
Sus scrofa	XP_005652858.1
Equus asinus	XP_014706509.1
Canis lupus familiaris	XP_003432370.1
Pan troglodytes	XP_001154136.1
Homo sapiens	NP_004611.1
Rattus norvegicus	NP_001101224.1
Mus musculus	NP_033450.2
Taeniopygia guttata	XP_002198512.2
Anas platyrhynchos	XP_005011386.1
Meleagris gallopavo	XP_010709560.1
Gallus gallus	XP_004941605.1

Cell treatment with poly(I:C) and poly(dA:dT)

To explore the effect of different stimuli on the expression of *chTRAF6 in vitro,* CEFs were first transfected using Lipofectamine 2000 (Invitrogen, USA), respectively, with poly(I:C) and poly(dA:dT) (InvivoGen, USA) according to the manufacturer protocol in a concentration-dependent manner, leaving uninfected cells as a control. Then, CEFs were treated with poly(I:C) and poly(dA:dT) in a time-dependent manner using the optimal concentration determined, respectively. Cells were harvested for total RNA extraction using TRIZOL reagent (Invitrogen) at the indicated time points.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissues and CEFs with TRIZOL reagent (Invitrogen), according to the manufacturer protocol. Total RNA was incubated with RNase-free DNase I (OMEGA, USA) for 45 min at 37°C. First-strand cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit with a gDNA Eraser (TaKaRa, China) according to the manufacturer instruction.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to determine the transcript level of *chTRAF6*. Each qRT-PCR mixture comprised 5 mL SYBR Green PCR master mix (TaKaRa), 3 mL nuclease-free water, 1 mL cDNA, and 0.5 mL each gene-specific primer (10 mM; Table 1). The cycling protocol consisted of 3 min at 98°C for pre-incubation, then 40 cycles for 10 s at 98°C, 30 s at 60°C, and 15 s at 72°C. Relative gene expression was normalized using the endogenous control gene *GAPDH* and was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008). Melting curve analysis verified the specificity of the products.

Statistical analysis

All analyses were performed using SAS 9.3 (SAS Institute Inc., USA). The data were plotted and values are reported as means ± standard error with GraphPad Prism 5 software (La Jolla, USA). P values < 0.05 were considered significant.

RESULTS

Sequence analysis of *chTRAF6*

We amplified *chTRAF6* cDNA by RT-PCR. The full-length coding sequence of *chTRAF6* (**Figure S1**) was found to be 1638 bp in length, and encoded a predicted 545-amino acid residue protein. We predicted the structure of *chTRAF6* using the SMART program (http://smart.embl-heidelberg.de), which indicated that chTRAF6 protein consisted of a RING finger domain (70-108 aa), two zinc fingers (204-261 aa), a coiled-coil region (314-371 aa), and a MATH domain (378-505 aa). Multiple sequence alignments revealed that *chTRAF6* shares high sequence identity with *TRAF6s* from other species (Figure 1), but shares a relatively high level of identity with those of other birds (84-98%), with the highest identity found with *Meleagris gallopavo* (98%) and *Anas platyrhynchos* (96%). Based on the deduced amino acid sequence of *TRAF6* genes, a phylogenetic tree was constructed to identify their evolutionary relationships. As shown in Figure 2, *TRAF6* genes in birds clustered in one group, which was divided into two clades. *G. gallus* was grouped into a cluster along with *M. gallopavo* and showed a particularly strong evolutionary relationship with *Taeniopygia guttata* and *A. platyrhynchos.*

Tissue distribution of *chTRAF6* mRNA

The tissue distribution of *chTRAF6* was determined by qRT-PCR. As shown in Figure 3, *chTRAF6* was broadly expressed in all examined tissues with the highest expression observed in the lung, followed by the spleen, pancreas, thymus, small intestine, brain, kidney, and liver. Relatively low expression levels were observed in the brain and heart, and the lowest expression was found in the leg muscle.

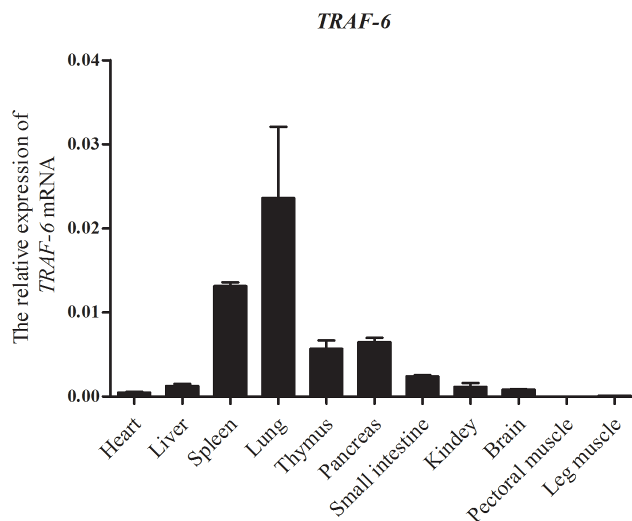

Figure 3. Tissue distribution of the *chTRAF6* gene in healthy chickens, as determined by qRT-PCR analysis. The values are reported as means ± SD (N = 3).

Expression of *chTRAF6* in immune organs of NDV-infected chickens

We determined *chTRAF6* expression levels in the immune organs of chickens following NDV F48E9 or Mukteswar infection to confirm whether *chTRAF6* was also associated with the innate immune response *in vivo*. As shown in Figure 4, we observed the *chTRAF6* gene to be significantly expressed in the bursa of Fabricius, spleen, and thymus of NDV-infected chickens.

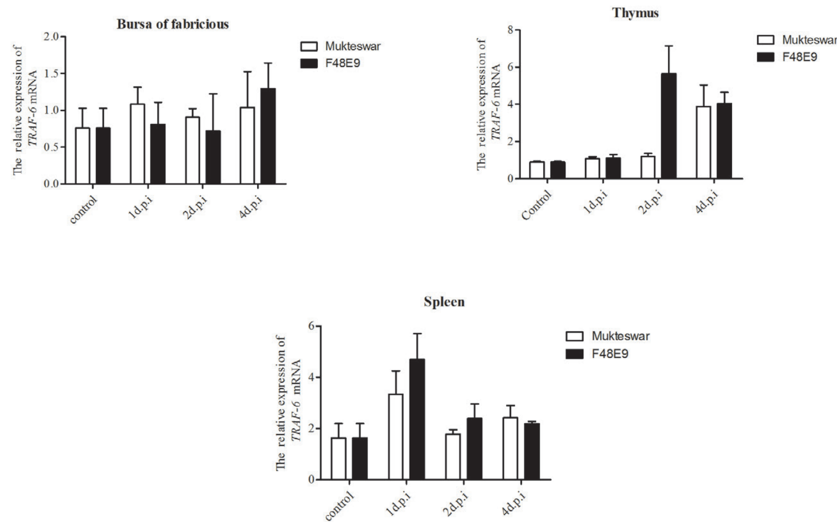

Figure 4. SPF chickens were infected with Newcastle disease virus (NDV), immune tissues including bursa of Fabricius, thymus and spleen were harvested at 1, 2, and 4 days post-infection, respectively. The values are reported as means ± SD (N = 3).

Differential expression of *chTRAF6* in NDV-infected CEFs

We also explored the effect of NDV infection on *chTRAF6* expression. Figure 5 shows that *chTRAF6* expression levels in CEFs sharply increased from 6 to 16 h and peaked at 16 h. Meanwhile, *chTRAF6* levels in Mukteswar-infected CEFs were significantly higher than in F48E9-infected CEFs.

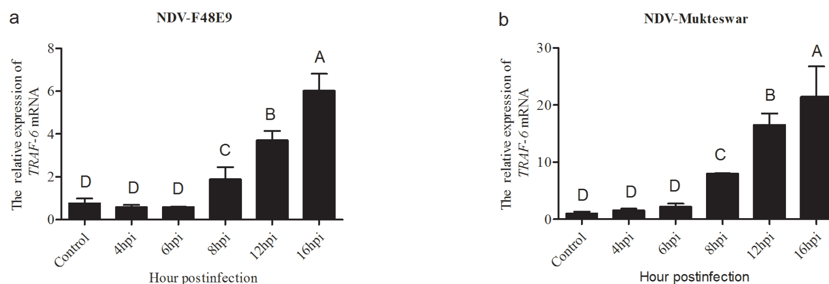

Figure 5. Expression of *chTRAF6* in chicken embryo fibroblast cells (CEFs) following NDV infection. Cells were harvested at the specified time points. Different letters above bars indicate a significant difference ($P < 0.05$). The values are reported as means ± SD (N = 3).

Expression of *chTRAF6* in CEFs after poly(I:C) and poly(dA:dT) challenge

We investigated whether the *chTRAF6* gene was involved in the immune response of CEFs following infection with viral analogues. As shown in Figure 6a, the expression of *chTRAF6* in CEFs significantly increased from control to 0.5 mg/mL poly(I:C), then decreased thereafter, reaching maximum expression at 0.5 mg/mL poly(I:C). An increase in the concentration of poly(dA:dT) tended to increase the expression of *chTRAF6* in CEFs, which peaked following transfection with 4 mg/mL poly(dA:dT) (Figure 6b). Then, we examined the expression levels of *chTRAF6* in CEFs subjected to poly(I:C) or poly(dA:dT) transfection at different time points using the optimum concentration, as described above. The expression levels of *chTRAF6* were significantly decreased from 3 to 9 h in CEFs transfected with poly(I:C) (0.5 mg/mL), reaching maximum levels at 3-h post-transfection (Figure 6c). Furthermore, the expression of *chTRAF6* in CEFs gradually increased, beginning 3 h after transfection with poly (dA:dT) (4 mg/mL) and peaking at 9 h (Figure 6d).

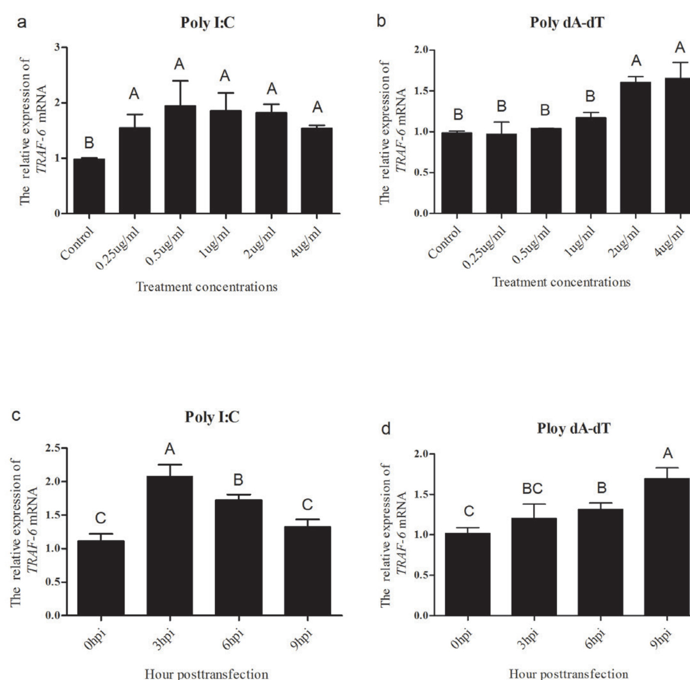

Figure 6. Expression of *chTRAF6* in CEFs following poly(I:C) or poly(dA:dT) infection. **a. b.** Cells were treated with increasing concentrations of poly(I:C) or poly(dA:dT). **c. d.** Cells were harvested at the specified time points. Different letters above bars indicate significant difference ($P < 0.05$). The values are reported as means ± SD (N = 3).

DISCUSSION

TRAF6 is an adaptor molecule that functions as an E3 ubiquitin ligase and is essential for multiple signaling pathways, such as TNFR, TLR, and IL-1R (Poole et al., 2006). In this study, we cloned and identified the gene sequence of *TRAF6* from chicken, which encoded a protein consisting of 545-amino acid residues. Results from BLAST analysis revealed the

gene to have high nucleotide and amino acid sequence similarity to *TRAF6* from other species, with the highest identity shared with *G. gallus*. In mammals, *TRAF6* contains a RING finger, five zinc fingers, a coiled-coil region, and a MATH domain (Ishida et al., 1996). SMART analysis revealed that *chTRAF6* possesses characteristics typical of TRAFs, with a RING finger domain, two zinc fingers of the TRAF-type, one coiled-coil region, and a MATH domain. There was high similarity between the gene sequence and the structural domains, suggesting *chTRAF6* may have a function similar to that of other mammalian TRAF6s. The intact RING finger domain, together with the first zinc finger of *TRAF6*, is essential for auto-ubiquitination and interaction with Ubc13 (Lamothe et al., 2008). However, whether the reduced number of zinc fingers affects the function of *chTRAF6* remains to be determined.

The phylogenetic tree based on the deduced amino acid sequences indicated that *G. gallus TRAF6* was grouped into the bird cluster, and *chTRAF6* showed a particularly strong evolutionary relationship with *M. gallopavo TRAF6*. Q-PCR analysis detected *chTRAF6* in all tissues examined, indicating ubiquitous and constitutive expression of this gene. The most abundant *chTRAF6* expression was found in the spleen, lung, thymus, and pancreas. In humans, the highest *chTRAF6* expression was detected in the thymus, testis, and epidermis (Zapata et al., 2000). Previous studies suggested that TRAF6 is a crucial cytoplasmic adaptor molecule responsible for the pathogen-recognition receptors (PRR)-mediated innate immune response (Bradley and Pober, 2001). The tissue distribution of *TRAF6* is an important determinant of its function, and can influence the capacity of cells to detect different microorganisms during different periods in different tissues (Cheng et al., 2015). The tissue distribution of *TRAF6* has been previously examined in humans (Starczynowski et al., 2011), mice (Mason et al., 2004), and ducks (Zhai et al., 2015). Different species exhibit different tissue expression profiles of *TRAF6*; abundant expression of *TRAF6* was observed in the spleen, which is the largest lymph organ, in humans, mice, and ducks, suggesting that this protein might be a critical factor for the immune system. *chTRAF6* mRNA was highly expressed in immune-related tissue, including bursa of Fabricius, spleen, and thymus, indicating a functional TRAF6-mediated innate immune response to pathogenic challenges in chickens (Stockhammer et al., 2010).

NDV is a negative-strand RNA virus, which can cause great economic losses in the global fowl industry (Kapczynski et al., 2013). NDV is recognized primarily by RIG-I in human and murine cells (Kato et al., 2006). Although RIG-I is not found in chickens (Barber et al., 2010), infection with NDV can trigger the expression of type I IFNs, cytokines, and several IFN-stimulated genes in the spleen, macrophages, splenic leukocytes, and embryo fibroblasts (Rue et al., 2011), indicating the presence of an unknown viral sensor that compensate the functions of absence RIG-I.

In this study, we choose 2-week-old chickens to infect with NDV, because of their effective immune systems. We explored whether the *chTRAF6* gene was expressed in CEFs and in immune organs of chickens following NDV infection. We demonstrated that *TRAF6* expression significantly increased both *in vivo* and *in vitro* upon infection with NDV, suggesting an antiviral role for chTRAF6 in chickens. Taken together, these data indicate that *chTRAF6* might function as a crucial protein in viral detection or in the activation of antiviral innate immune responses in chickens.

The innate system provides the first line of host defense against invading pathogens (Barbalat et al., 2011). Host cells express PRRs, which are engaged by pathogen-associated molecular patterns (PAMPs), thus triggering the innate immune response, leading to rapid production of type I IFNs (Takeuchi and Akira, 2010).

Viral RNA and DNA can trigger immediate antiviral responses in human and mouse cells (Ablasser et al., 2009). Poly(dA:dT) does not favor the assembly or stability of nucleosomes *in vitro*. Poly(I:C) and poly(dA:dT) are synthetic analogs of viral nucleic acids, which trigger type I IFN responses in mammals (Gitlin et al., 2006), and dsRNA has long been considered a candidate viral PAMP. Treatment of mammalian cells with poly(I:C), which is a synthetic double-stranded RNA, induces type I IFN production via the activation of the transcription factors IRF-3 and NF-kB (Levy and Marié, 2004). Expression of *TRAF6* in mammalian cells has been demonstrated in previous studies, showing that TRAF6 plays a crucial role in dsRNA-triggered antiviral signaling pathways (Mao et al., 2010). In our study, the expression of *chTRAF6* was significantly induced in CEFs transfected with poly(I:C), which is recognized by MDA5 (Hayashi et al., 2014), suggesting that *chTRAF6* is essential for the induction of effective innate immune responses. In addition, poly(dA:dT) is a repetitive synthetic double-stranded DNA, which is also indirectly detected by RIG-I (Thompson et al., 2011), and *chTRAF6* expression was induced in CEFs following treatment with poly(dA:dT) in the present study. These results support the presence of potentially unknown DNA sensors in chicken cells that can detect poly(dA:dT) motifs. In addition, in contrast to poly(I:C), the expression levels of *chTRAF6* followed a gradual upward trend in CEFs treated with poly(dA:dT). However, the mRNA and protein expression levels may exist in inverse proportions. To further verify the function of this gene, the expression levels of *chTRAF6* should be quantified by western blotting. Taken together, these results indicate that infection with RNA and DNA viruses activates different antiviral pathways, and that chTRAF6 plays a crucial role in chicken defense against RNA and DNA viral infection.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Figure S1. Nucleotide and deduced amino acid sequences of *chTRAF6*. The stop codon is indicated by an asterisk.