



Molecular characterization, tissue distribution and expression analysis of *TRIM25* in *Gallus gallus domesticus*



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ABSTRACT

TRIM25, a member of the tripartite motif-containing (TRIM) family of proteins, plays an important role in cell proliferation, protein modification, and the RIG-I-mediated antiviral signaling pathway. However, relatively few studies have investigated the molecular characterization, tissue distribution, and potential function of *TRIM25* in chickens. In this study, we cloned the full-length cDNA of chicken *TRIM25* that is composed of 2706 bp. Sequence analyses revealed that *TRIM25* contains a 1902-bp open-reading frame that probably encodes a 633-amino acid protein. Multiple comparisons with deduced amino acid sequences revealed that the RING finger and B30.2 domains of chicken *TRIM25* share a high sequence similarity with human and murine *TRIM25*, indicating that these domains are critical for the function of chicken *TRIM25*. qPCR assays revealed that *TRIM25* is highly expressed in the spleen, thymus and lungs in chickens. Furthermore, we observed that *TRIM25* expression was significantly upregulated both in vitro and in vivo following infection with Newcastle disease virus. *TRIM25* expression was also significantly upregulated in chicken embryo fibroblasts upon stimulation with poly(I:C) or poly(dA:dT). Taken together, these findings suggest that *TRIM25* plays an important role in antiviral signaling pathways in chickens.

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1. Introduction

Tripartite motif-containing (TRIM) proteins are defined by the presence of an N-terminal RBCC motif, comprising a Really Interesting New Gene (RING) finger domain, one or two B-boxes, and a coiled-coil region (Meroni and Diez-Roux, 2005). The RING finger domain is a specialized zinc-binding motif that binds two zinc atoms. Moreover, this domain has E3 ubiquitin ligase activity and mediates ubiquitination events (Joazeiro and Weissman, 2000). The B-box domain, which occurs after the RING finger domain, is also a zinc-binding motif. The B-box domains of proteins can be classified into two types, B-box1 and B-box2. Metal binding titration experiments and structural nuclear magnetic resonance spectroscopy have revealed that B-box2 binds to one zinc atom. However, whether B-box1 also coordinates zinc atoms remains unknown (Borden et al., 1993, 1995). The B-box domain is followed by

the coiled-coil region, which mainly mediates the formation of high molecular weight complexes through interactions with other proteins (Reymond et al., 2001). Although TRIM proteins have been identified in many species, the number of TRIM proteins varies among different species. The TRIM family of proteins function as important E3 ubiquitin ligases and are involved in various biological processes, including cell differentiation, apoptosis, transcriptional regulation, signaling transmission, and host innate immune responses.

Human *TRIM25* was first identified as an estrogen-responsive finger protein, and its expression is induced in human breast cancer cells upon treatment with estrogen (Inoue et al., 1993; Ikeda et al., 1997, 2000). Moreover, *TRIM25* expression is also induced in immune cells following treatment with types I and II interferons (IFNs), owing to the presence of an IFN-stimulated response element in the first intron of the human *TRIM25* gene (Nakasato et al., 2006; Rajsbaum et al., 2008; Carthagena et al., 2009). *TRIM25* functions as an E3 ubiquitin ligase to regulate cell proliferation by targeting the negative cell cycle regulator 14-3-3 σ for proteolysis (Urano et al., 2002). *TRIM25* also conjugates with ISG15 to modify itself and 14-3-3 σ in a RING domain-independent manner (Nakasato et al., 2006; Zou and Zhang, 2006; Zou et al., 2007). Recently, it was reported that *TRIM25* potentiates the retinoic acid-inducible gene-1 (RIG-I)-mediated antiviral signaling pathway by delivering a Lys 63-linked polyubiquitin moiety to the Lys 172 residue of RIG-I upon viral infection, thereby enabling efficient interaction with the mitochondrial antiviral-signaling (MAVS) protein, leading to

Abbreviations: *TRIM25*, tripartite motif protein 25; ORF, open-reading frame; RING, Really Interesting New Gene; IFN, interferon; SPF, specific pathogen-free; UTR, untranslated region; NDV, Newcastle disease virus; RIG-I, retinoic acid-inducible gene-1; MDA-5, melanoma differentiation associated gene 5; MAVS, mitochondrial antiviral signaling; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Poly(I:C), polyinosinic–polycytidylic acid; Poly(dA:dT), poly(deoxyadenylic–deoxythymidylic); Dpi, days post-infection; CEFs, chicken embryo fibroblasts; MOI, multiplicities of infection; RACE, rapid amplification of cDNA ends; Df, degrees of freedom.

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IFN production (Gack et al., 2007; Zeng et al., 2010). In addition, the linear ubiquitin assembly complex specifically suppresses RIG-I-mediated antiviral activity by binding to human TRIM25 and inducing its degradation (Inn et al., 2011). These studies indicate that TRIM25 is the pivotal component in the RIG-I-mediated antiviral signaling pathway.

TRIM25 genes have been identified and characterized in humans, mice, and rats in several previous studies (Inoue et al., 1993; Orimo et al., 1995; Inoue et al., 1999). However, relatively few studies have investigated the sequence, tissue distribution, and function of chicken *TRIM25*. Barber et al. (2010) reported that the absence of RIG-I in chickens resulted in increased susceptibility to avian influenza virus compared to ducks. This observation compelled us to investigate whether *TRIM25* is present in chickens and whether it is involved in the antiviral immune response. In this study, we cloned the full-length cDNA of chicken *TRIM25* and investigated the tissue distribution of *TRIM25* mRNA. Furthermore, we analyzed changes in *TRIM25* expression levels both in vitro and in vivo in chickens following infection with Newcastle disease virus (NDV), and in chicken embryo fibroblasts (CEFs) stimulated with polyinosinic-polycytidylic acid (poly[I:C]) or poly(deoxyadenylic–deoxythymidylic) acid (poly[dA:dT]).

2. Materials and methods

2.1. Chicken and tissue sample collection

Specific pathogen-free (SPF) chickens were purchased from the Beijing experimental animal center (Merial Inc., Beijing, China) and housed at the experiment farm of Sichuan Agricultural University (Sichuan, China). All protocols used in this study were approved by the animal ethics committee of Sichuan Agricultural University. Three chickens were killed at the age of 3 weeks. Tissue samples, including the heart, liver, lungs, spleen, thymus, pancreas, small intestine, kidney, brain, pectoral muscle, and leg muscle of each chicken were harvested and immediately snap frozen in liquid nitrogen and then stored at -80°C .

2.2. NDV challenge and sample collection

Because the immune system does not fully develop at one-week-old young chickens, we choose two-week-old chickens for infection with NDV. Twenty two-week-old SPF chickens were randomly divided into uninfected and infected groups. Each of 10 chickens in the infected group was infected intraperitoneally with a total dose of $10^{6.0}$ 50% embryo infectious dose of NDV in 0.1 mL. The uninfected group was mock-infected with phosphate-buffered saline as a control. At 2 days post-infection (dpi), five infected and control-uninfected chicks were euthanized, respectively. Immune tissues, including the spleen, thymus, and bursa of Fabricius, of every killed chicken were collected, snap-frozen in liquid nitrogen, and then stored at -80°C until thawed for RNA isolation.

2.3. Cell culture, virus infection and transfection of poly(I:C) and poly(dA:dT)

CEFs were prepared from 9-day-old SPF chicken embryos and cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA), 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Infection of cells with different multiplicities of infection (MOI) of NDV was performed by incubating 500 μL of diluted NDV at 37°C for 1 h. The supernatant of the diluted virus was replaced with DMEM with 2% FBS, and then incubated for 12 h before cell collection.

The double-stranded RNA and DNA used in our study were poly(I:C) and poly(dA:dT), which were purchased from InvivoGen (San Diego, CA, USA). Transfection of poly(I:C) and poly(dA:dT) was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA)

according to the manufacturer's instructions. The poly(I:C), poly(dA:dT), and transfection reagent were diluted with DMEM, respectively. After 5 min, the diluted poly(I:C) and poly(dA:dT) was combined with the diluted transfection reagent, incubated for 20 min, and then added to cell supernatants and incubated for 4 h. The cell supernatant was replaced with DMEM supplemented with 10% FBS and then incubated for 9 h before cell collection.

2.4. Total RNA extraction and reverse transcription

Total RNA was isolated from collected tissues and CEFs using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Extracted RNA was dissolved in 40 μL of RNase-free water and stored at -80°C until thawed for reverse transcription.

RNA integrity and purity was assessed by visual inspection using NanoVue Plus™ spectrophotometer (GE Life Science, Piscataway, NJ, USA) after being electrophoresed in formaldehyde gel. Only RNAs with an A260/A280 ratio between 1.9 and 2.1 were used for cDNA synthesis. Reverse transcription of total RNA was carried out using the PrimeScript™ RT Reagent Kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The reaction was performed in a total volume of 20 μL containing 4 μL of $5\times$ PrimeScript™ Buffer, 1 μL of PrimeScript™ RT Enzyme Mix I, 1 μL of Oligo dT Primer, 1 μL of random hexamers, 11 μL of RNase-free water, and 1 μg of total RNA. The reaction mixture was incubated at 37°C for 15 min, and 85°C for 5 s. The cDNA was stored at -20°C until used for quantitative real-time PCR (qPCR).

2.5. Cloning and sequencing of the full-length cDNA for chicken's *TRIM25* gene

According to the predicted *TRIM25* gene mRNA sequence of *Gallus gallus* (accession no.: XM_415653.4) retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>), a pair of primers (Table 1) was designed to amplify the full coding region of chicken *TRIM25* mRNA from the spleen cDNA. The PCR products were separated by electrophoresis on a 3% agarose gel and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The purified fragments were cloned into the pMD-19T vector, and five clones were selected for sequencing.

We further performed rapid amplification of cDNA ends (RACE) to determine the 5'- and 3'-UTR of chicken *TRIM25* cDNA. The 5' and 3' RACE cDNA were synthesized from spleen RNA using the SMART™ RACE cDNA amplification Kit (Clontech, USA) according to the

Table 1
Primer sequences for cloning and real-time PCR.

Gene name	Primer sequence (5' → 3')	
<i>TRIM25</i>	F: ATGGCGACGTTGACCAAG R: TTACCAGAGAGAGAACAGT	Coding region amplification
5'-RACE	TGCAGACAAGTCTGCCAGTAGATG CAGGTTTGCTCCAGGCAGTAGGC	5'-first nested PCR 5'-second nested PCR
3'-RACE	CCGCCTGGGTAGGAACAGCAGTT GATGGAGGGTTCGTCTTTCTTGAC	3'-first nested PCR 3'-second nested PCR
<i>TRIM25</i>	F: GAGGAGACAGATACCCAAGACA R: CAGAGCCACTTTGCTGTGAG	Real-time PCR
<i>IFN-α</i>	F: CAGGATGCCACTTCTCTCAC R: AGGATGGTGTGTTGAAGGAG	Real-time PCR
<i>IFN-β</i>	F: CCTCAACCAGATCCAGCATTAC R: CCCAGGTACAAGCACTGTAGTT	Real-time PCR
<i>GAPDH</i>	F: AGGACCAGGTTGTCTCTGT R: CCATCAAGTCCACAACCGG	Real-time PCR
<i>β-actin</i>	F: GAGAAATGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	Real-time PCR

manufacturer's instructions. Two sets of gene-specific primer (Table 1) were designed to amplify the 5'- and 3'-UTR of chicken TRIM25 mRNA based on the sequenced cDNA fragment, respectively. The PCR products of 5' and 3' RACE were separated by electrophoresis on a 3% agarose gel and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). The purified fragments were cloned into the pMD-19T vector (Takara Bio, Inc.), and five to ten clones were selected for sequencing.

2.6. qPCR analysis of chicken TRIM25, IFN-α and IFN-β mRNA

Relative expression levels of chicken TRIM25, IFN-α and IFN-β mRNA were measured by qPCR using a CFX-96 qPCR thermal cycle instrument (Bio-Rad, Hercules, CA, USA). A dilution series of the standard was used

to calibrate each plate in the qPCR assay. Reactions were performed in a volume of 10 μL, which included 1.0 μL of cDNA preparations, 0.5 μL of each specific primer, 5 μL of Ssofast EvaGreen supermix (Bio-Rad), and 3.0 μL of ddH₂O. The gene-specific primers used in this study are presented in Table 1. The optimum thermal cycling conditions consisted of an initial denaturing step at 98 °C for 2 min, 39 cycles of 98 °C for 2 s, and at the optimal annealing temperature of each primer pair for 20 s. The specificity of amplification was checked by melting curve analyses and 1.5% agarose gel.

2.7. Statistical analysis

To investigate the tissue distribution of TRIM25 mRNA in chicken, we chose glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-

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GCAGCCGGACGGCAGCAGCGGGAGA
26 ATGGCGACGTTGACCAAGCTCAGTCGGAGCCGAACTCTGTTGGCGTGGAGGAGGACCTGACGTCTCCATCTGCCTCAGCATCTTCGAC
1 M A T L T K A Q S E P N L L A L E E D L T C S I C L S I F D
116 GCCCCCGTGACGGTGCCCTGCGGGCACAACCTTCTGTGCCCTACTGCGTGGAGCAAACTGGGCCAGCCAGGTGCGGGACTTCAGCTGCCCG
31 A P V T V P C G H N F C A Y C L E Q T W A S Q V R D F S C P
206 CAGTGCCGCACCACCTTCCCGGACCGCCCGCAGCTCCACAAGAACACGGTGCCTGTCGCGGGTGGTGGAGCAGCTGCAGGGCTGCGGGGAC
61 Q C R T T F P D R P Q L H K N T V L C R V V E Q L Q G C G D
296 GGAAGGAGGACGATGAGCACGAGGAGGAAGAGCAGGAGGCGGACGCCCATCTACTGCGACAGTTGCTCTCAAGCGGGCCGACAGCAG
91 G K E D E D E H E E A A A G C Q E A A A P I Y C D S C L A A A T Q
386 ACCTGCCTCACCTGCATGGCTCCTTCTGCCCGAGCAGCTGCGGCGCAGTACGACAGCCCGGCTTCCGCCACCATCAGCTGTCGCCG
121 T C L T C M A S F C P E H L R P H Y D S P A F R H H Q L C P
476 CCGTGCGGGACCTGCAGCAGAGGAAGTGCCCGCAGCACAAAGCTCTTTGAGTTCTTCTGCAGCCAGCAGCTGCTGCATCTGCTCG
151 P V R D L Q R K C P Q H N K L F E F F C S Q H S C A C I C S
566 CTTGCGCTGTAGCCACAAGCTGTGCCACACCGCCGCTGCGCAGCAGGCCAAAGACAACCGCTGAGTCAAGACTGCAAGAGGCTGGCA
181 L C L L S H K L C H T S P L Q Q A K D N A E S A L K K R L A
656 GAGCTGCACAATCAGAGTAAAAGATCTGTGCAAGCAATGAACTCTGTGAAAACAATCCAAAGACAAGCTGCTGAGACAGCTGCCAGAAAAG
211 E L H N Q S E R S V Q A A M N S V K T I Q R Q A A E T A A R K
746 CGAGATTTGCTGAGCTGTTTTTTGAAATTAAGCTTTAATTGAGAAAAGAAAACCAGACCCCTAAAAGTATTTTGAAGAGAA
241 R D L L R A E F L E I K A L I E E K E N Q T L K V F M E E E
836 AAAAGAGTTGCAATAAGTTTGATTACGTATATAAAATTTGGGAAATAAGAAAGAAATTCAGTCTCTGAGAGACCAGATTGAGATG
271 K R V C N K F D Y V Y K I L G N K K N E I Q S L R D Q I E M
926 GCACTGACTGAAGGTGATGACGTTCTCTTTTAAAGAGAGCAGCAGCGCTGCAACGAACATCAATAAAAAGAGGCTTTTGTCCAGTAATT
301 A L T E G D D V L F L K R A A A L Q R T S I K E A F V P V I
1016 GAAATGGACAACAACATGATACATGCTGCTTATCAGTCTGCCATTAACCTTAAAGATGTTGTCAAACCTGTCAGTGAATGTGCTGTGGAT
331 E M D N N M I H A A Y Q S A I N L K D V V K L A V N V S V D
1106 AAAAGAACGGATGCAAAAACCACCTGGGAAAAACAAGCCTCCTTCAGTGCCTTCCCAAAACAACCCGTTGTAAAAAAGAGCTTGCT
361 R K T D A K P P G K T K P P S V P S P N K P V V K K L A
1196 GCAGACTCAGTACCACAAAAGACAAAATCCCTCACCAACTAAAACCACTCTGCTGGAGGAGACAGATACCAAGACAAAAGGAATCCT
391 A D S R T H K D K I P H P T K T T L L E E T D T Q D K R N P
1286 GTTAAACTTGACCAAGCATGGGAGCACAAGCATGGGAGCACAAGCGCTGCAAAATGCTGCTAAAGTAAAAGAACTTATTAGCAGCTTC
421 V K L A P S M G A P S M G A P S A A N A A K V K E L I S T G
1376 CTGAAAAAACCAGAGCGGAGCTTTTGCAGTATGCTGCTAACGTCACGCTGGATTTCAAACACAGCTCACAGCAAAGTGGCTCTGCTGAG
451 L K K P R A E L L Q Y A A N V T L D F N T A H S K V A L S E
1466 AGATACCAAGATGCTGTTTCAGACACCAACTGAATTACAACCACCACCTCAGCGTTTCACCGACTGCCCCAGGTGCTGGGGTTC
481 R Y T K M S V S D T Q L N Y N H H P Q R F T D C P Q V L G F
1556 CAGTGCTTCAAGAGAGGCATCCACTACTGGGAAGTGGAAAATGCAGCAGCAACAACCTTCTGTGGCATTGGCATTGCTATGGCAGCATGGAG
511 Q C F K R G I H Y W E V E M Q Q N N F C G I G I C Y G S M E
1646 CGGCAGGGGCGGACAGCCGCTGGGTAGGAACAGCAGTTCTTGGTGATCGAGTGGTTAATTCAAAAATTCAGCTGGCATAATGAT
541 R Q G P D S R P L G R N S S S W C I E W F N S K I S A W H N D
1736 GTTAAAAGTGCTTACCAATACAAAAGGCCACTAAGATTGGTGCTGCTCCACTGTGATGGAGGGTTCGTGCTTTTCTGACTGTGAG
571 V E K C L P N T K A T K I G V L L H C D G G F V L F L T V E
1826 GAAAAGCTTAATTTGATCTATAAAATTCAAAAGCCAGTTTACTGAAGCTGTGTACCCTGCCCTTCTGGTTATTTTCCAGTGGCAGCTGTCTC
601 E K L N L I Y K F K A Q F T E A V Y P A F W L F S S G T V L
1916 TCTCTGGTAAATGAAACCATAAGGCTGGTGCTTACGTAATTTGCTTGAGTACTGACATGCAGGTAATCACTCCTTGCAACATCTC
631 S L W *
2006 ATCTTTGTAGCTATAAAATGATGTTGAAATCATAAAACAATTTACATGCTTGTCTTAGACTAAGCAGTATCAAAGATTCTGGGTTGGTTG
2096 GGTTTTTTTTTTGGTGGGTTTTGGTGTTTTCTTTTCAATGAAAATTCATGACTCTTTTAGGTAGTTTAGGATATTGCA
2186 TCATTCTATGGCCTGATGCTAAAGCCTTTAGACAGTGATTTAGTAAAACGATGACAGTAAAACCCACAGAGAAAAGGATTTGCAAAAGC
2276 TACACCGTCTTCCAAAAGCAAAGTTATGACACAACCCACTAGCACATGCCCTCAGTAGCTGTAGATTCTGCAGTTGCTGTAAAGG
2366 TCTCCTGTGTTTACAGGTAGCAATATGACTGTTTGAAGAGGAAATAGGAGAGATTTCCCTGAAAAGGTGTTTCAGGTTTGGAGTGGTT
2456 TCAGTTTTGCATTAACCTTTTCTCTATATTCAGACTGTTGCTAAAGCCAGGATTTACAATGTGTCTCTCAGTGTGCAATGGCAGCTGA
2546 GATGGAGCAGAAATGAACTGTTGAAAGAGGTGAATTCAGGCTTGTGACACTCAGTTGTAATAATCACGATGACAAAACCTCATTAGAGACT
2636 TTCAGAGCTGATGATAAGACATGTATCAGAATCACCGGAAAATAAATGACAGTGAATATAAAAAAAAAA

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Fig. 1. The full-length of chicken TRIM25 cDNA sequence and the deduced amino acid sequence. The stop codon is shown as "*". The numbers refer to the nucleotide position.

actin (β -actin) as reference genes to determine the expression levels of *TRIM25* mRNA in different tissues. The expression level of *TRIM25*, *IFN- α* and *IFN- β* mRNA in CEF was calculated relative to the expression of *GAPDH*. All expression data were expressed as means \pm standard error ($M \pm SE$). The expression levels of *TRIM25* in vivo of chicken infected with NDV were subjected to *t*-test of SAS 8.0 software (SAS Institute, Cary, NC, USA) to identify differences between

control and NDV-infected groups. The expression level of *TRIM25*, *IFN- α* and *IFN- β* in CEF response to NDV, poly(I:C) or poly(dA:dT) were subjected into one-way ANOVA of SAS 8.0 software using the Tukey–Kramer method to identify differences between groups. *F*-values and degrees of freedom (df) in every statistics report were cited in results. Comparisons were considered significant at a probability (*P*) value < 0.05.

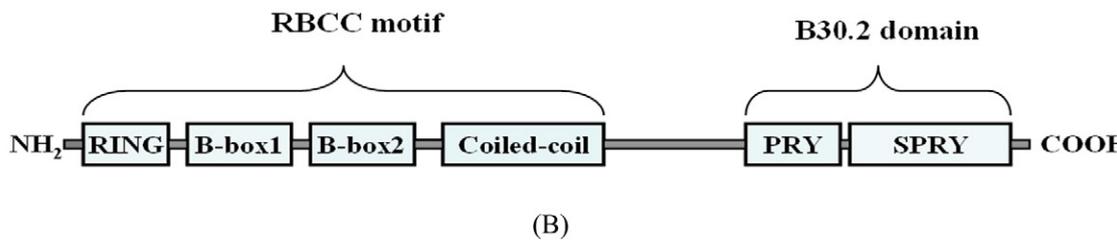
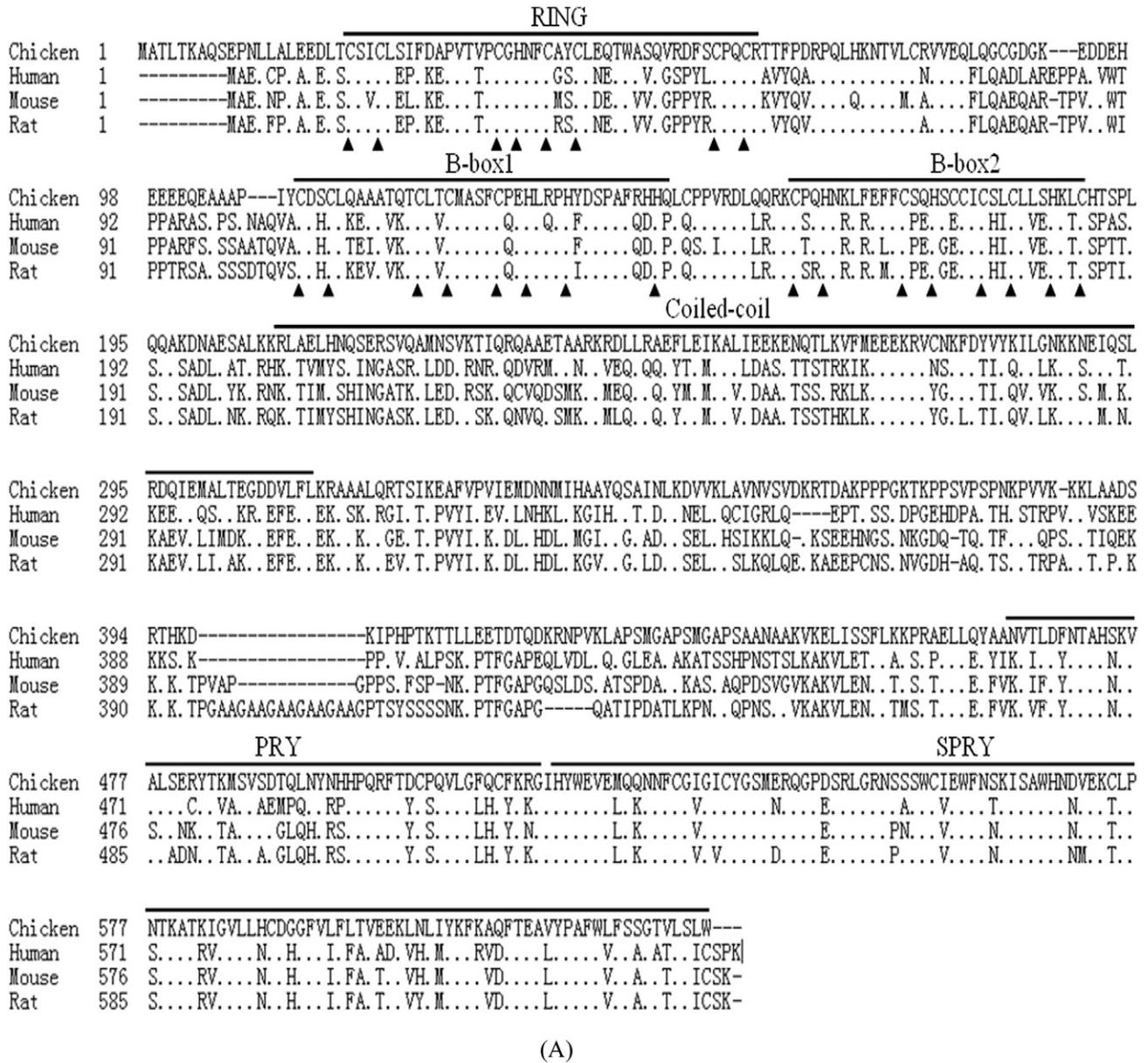


Fig. 2. Alignment and architecture of predicted amino acid sequence for chicken *TRIM25*. (A) Multiple comparisons of amino acid sequence of chicken, human, mouse and rat *TRIM25* proteins. Putative RBCC motif (including RING finger, B-box1, B-box2 and Coiled-coil domains) and B30.2 (PRY/SPRY) were indicated by bold lines. The numbers refer to the amino acid (AA) position. Identical AA residues are marked with dots. The triangles (\blacktriangle) denote the characteristic zinc-finger motif of RING domain and B-boxes. (B) A schematic diagram of domain architectures of predicted chicken *TRIM25* protein.

Table 2
The similarity of predicted chicken TRIM25 protein with other species.

Species	GenBank accession number	Full length amino acids	Identity (%)
<i>Gallus gallus</i>	XP_415653.4	672	100
<i>Meleagris gallopavo</i>	XP_003211618.1	547	78.16
<i>Homo sapiens</i>	NP_005073.2	630	47.59
<i>Mus musculus</i>	NP_033572.2	634	45.69
<i>Rattus norvegicus</i>	NP_001009536.1	643	46.49
<i>Pan troglodytes</i>	XP_511899.3	630	47.52
<i>Bos primigenius</i>	XP_548223.3	631	51.27
<i>Canis familiaris</i>	NP_001093806.1	631	47.43

3. Results

3.1. Cloning and sequence analysis of the chicken TRIM25 cDNA

The full-length chicken TRIM25 cDNA was found to be 2706 bp in length. It consisted of a 1902 bp ORF, preceded by 25 bp 5'-UTR and followed by a 779 bp 3'-UTR with a 11 bp poly(A) tail (Fig. 1). The chicken TRIM25 cDNA encoded a predicted 633 amino acid residues protein with an isoelectric point of 7.99 and a molecular weight of 71.42 kDa (http://web.expasy.org/compute_pi/). The obtained cDNA sequence was deposited in the GenBank database under the accession number KM879873.

Structural analysis of the predicted chicken TRIM25 protein using the SMART program (<http://smart.embl-heidelberg.de/>) revealed that chicken TRIM25 harbored a RBCC motif at the N-terminus (amino acids 21–311) and a B30.2 (SRY/SPRY) domain (amino acids 462–630) at the C-terminus (Fig. 2). The RBCC motif of chicken TRIM25 consisted of a RING finger domain (amino acids 21–66), B-box1 (amino acids 109–147), B-box2 (amino acids 160–190) and a coiled-coil region (amino acids 204–312). The RING finger domain of chicken TRIM25 has a conserved zinc-blend motif C-X₂-C-X₁₁₋₁₆-C-X-H-X₂-C-X₂-C-X₇₋₇₄-C-X₂-(CD) (C₃HC₄), which is a typical characteristic motif of RING finger domain present in human and murine TRIM25. The B-box1 and B-box2 of chicken TRIM25 contained similar but different zinc-binding motifs

C-X₂-C-X₇₋₁₂-C-X₄-C-X₂-(CH)-X₃₋₄-H-X₄₋₉-H and C-X₂-H-X₇₋₉-C-X₂-(CDHE)-X₄-C-X₂-C-X₃₋₆-H-X₂₋₄-(CH), respectively, which is consistent with the consensus sequences from B-box1 and B-box2 identified in human and murine TRIM25.

A comparison of the predicted amino acid sequence of chicken and other species of TRIM25 reveals homologies of 100%, 78.16%, 47.59%, 45.69%, 46.49%, 47.52%, 52.27%, and 47.43% for *G. gallus*, *Meleagris gallopavo*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Bos primigenius*, and *Canis familiaris*, respectively (Table 2). The phylogenetic tree based on the deduced amino acid sequences revealed that *G. gallus* TRIM25 was grouped into a cluster containing *M. gallopavo* TRIM25, but was phylogenetically separated from that of other mammalian species (Fig. 3).

3.2. Tissue distribution of TRIM25 mRNA

An expression profile of chicken TRIM25 was determined by qPCR using GAPDH and β -actin as housekeeping genes. As shown in Fig. 4, TRIM25 was ubiquitously expressed in all examined tissues with the most abundant expression levels in the spleen, lungs, and thymus, followed by the small intestine, pancreas, kidney, brain, liver, and heart, and relatively quite low expression levels in the pectoral and leg muscles.

3.3. TRIM25 expression is induced in vitro and in vivo following NDV infection

To characterize whether TRIM25 was involved in the antiviral immune response of the chicken, TRIM25 expression levels following infection with different doses of NDV were analyzed at 12 hours post infection (hpi). As shown in Fig. 5A, TRIM25 expression levels in CEFs significantly increased with increasing MOI of NDV from 0.01 to 1 ($F = 21.70$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$). TRIM25 expression levels in CEFs infected with an NDV MOI of 1 were significantly higher than that in the control CEFs and those infected with MOIs of 0.01 and 0.1 ($P < 0.05$), respectively. TRIM25 expression levels in CEFs infected with an MOI of 0.1 were also higher compared to that in control CEFs and

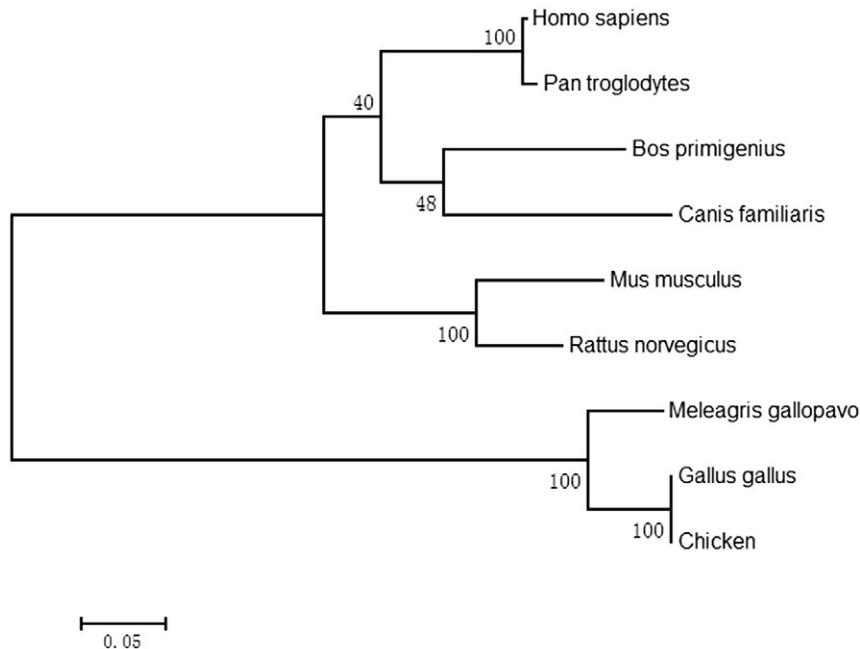


Fig. 3. Phylogenetic analysis of chicken TRIM25. A phylogenetic tree of was constructed by Mega5.0. The sequences were derived from the predicted amino acid sequences of chicken TRIM25 and the GenBank entries with accession numbers NP_033572.2 (*Mus musculus*), NP_001009536.1 (*Rattus norvegicus*), NP_005073.2 (*Homo sapiens*), XP_511899.3 (*Pan troglodytes*), XP_548223.3 (*Canis familiaris*), XP_415653.4 (*Gallus gallus*), NP_001093806.1 (*Bos primigenius*) and XP_003211618.1 (*Meleagris gallopavo*). The scale bar is 0.05.

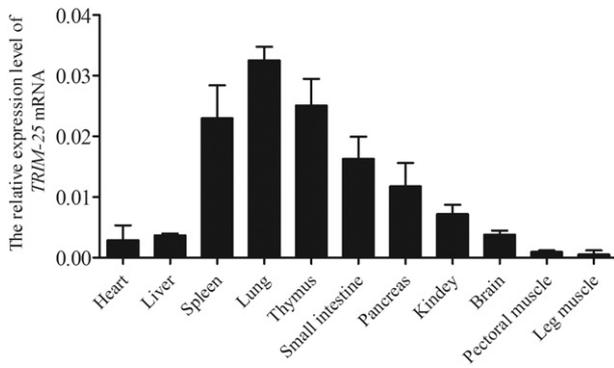


Fig. 4. Relative expression levels of *TRIM25* gene in different tissues of chicken were measured. Total RNA from different tissues of three chickens was used to perform the real-time PCR. Every sample was performed in duplicate. The expression levels of chicken *TRIM25* gene were normalized to the expression of *GAPDH* and β -*actin* genes. The values represent the mean \pm SD (n = 3).

those infected with an MOI of 0.01, but the difference among the three different MOIs were not statistically significant.

We next investigated *TRIM25* expression levels at different timepoints in CEFs infected with an MOI of 1 of NDV. The results showed that *TRIM25* expression levels in the CEFs increased sharply from 4 hpi

and peaked at 16 hpi ($F = 8.94$, $df_1 = 5$, $df_2 = 12$, $P < 0.05$) (Fig. 5B). Meanwhile, *TRIM25* expression levels in NDV-infected CEFs at 12 and 16 hpi were significantly higher compared to that at 0, 4 and 6 hpi ($P < 0.05$). Although *TRIM25* expression levels at 4, 6 and 8 hpi were higher than that at baseline, the difference among these timepoints were not statistically significant.

To establish whether *TRIM25* was also associated with antiviral response in vivo, we further measured *TRIM25* expression levels in the immune-related tissues of chickens following NDV infection (Fig. 5C). The results showed that *TRIM25* expression levels were also significantly upregulated in the spleen, thymus, and bursa of Fabricius of NDV-infected chickens at 2 dpi compared to those in uninfected-control chickens ($P < 0.05$). Taken together, these results suggest that *TRIM25* might function as an important protein involved in chicken antiviral response.

3.4. Induction of chicken *TRIM25*, *IFN- α* , and *IFN- β* in CEFs transfected with *poly(I:C)* and *poly(dA:dT)*

Viral RNA and DNA have been shown to immediately trigger antiviral responses in human and murine cells (Gitlin et al., 2006; Kato et al., 2006; Ablasser et al., 2009; Chiu et al., 2009). Therefore, we determined whether *TRIM25* was involved in the response of chicken cells to dsRNA and dsDNA stimulation. As shown in Fig. 6A, *TRIM25* expression levels in

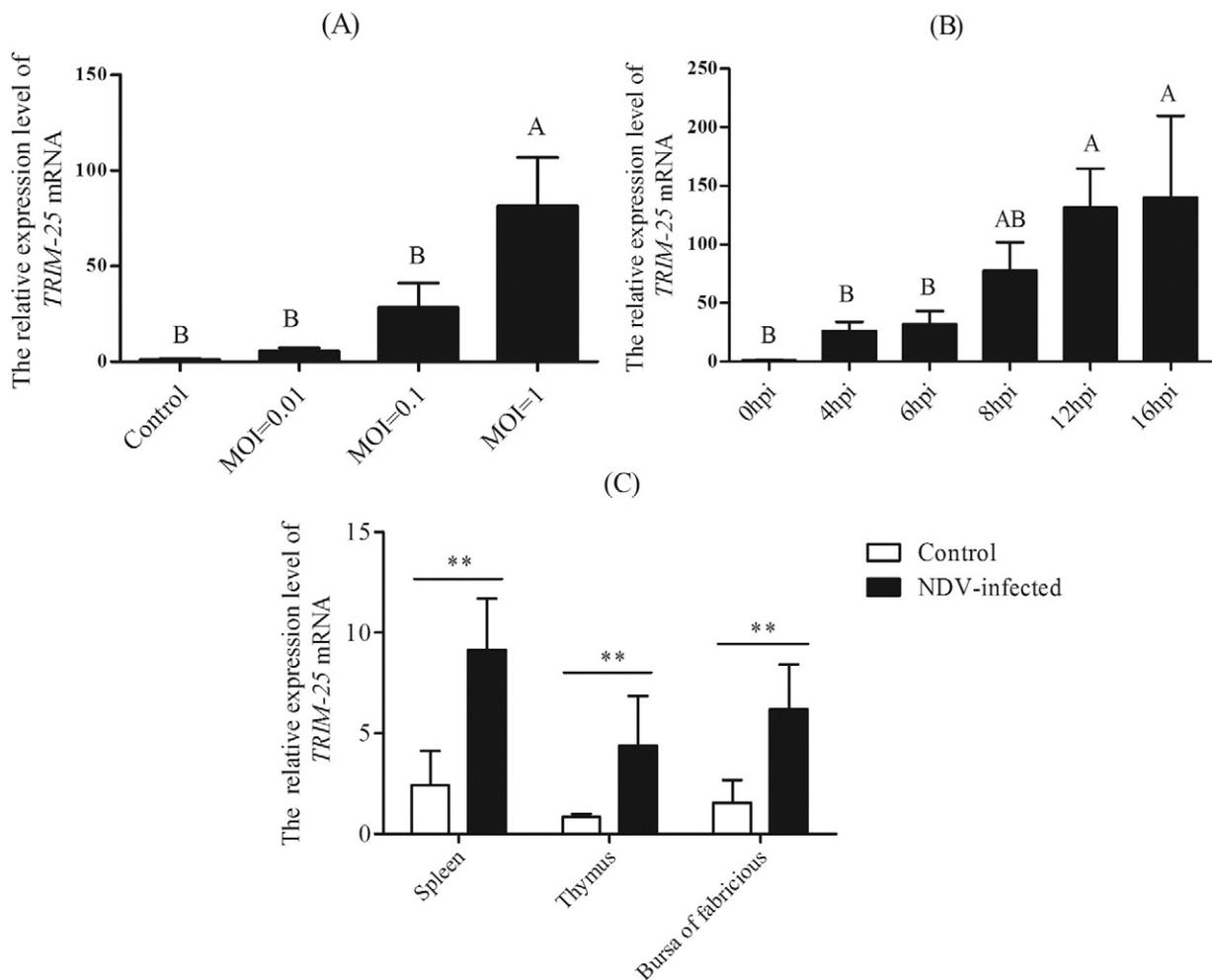


Fig. 5. The expression levels of *TRIM25* gene in CEF after infection with NDV. (A) CEFs were infected with different MOIs of NDV and then analyzed for the expression levels of *TRIM25* gene by real-time PCR. (B) After infection with NDV, the expression levels of *TRIM25* gene in CEF were analyzed at different points. Data represent fold increase relative to control-uninfected cell. (A) and (B) data shown are the mean \pm SD and subjected into one-way ANOVA to identify difference between groups. Columns sharing completely different letters show significant difference ($P < 0.05$). (C) SPF chicken were inoculated with NDV, specific tissues including spleen, thymus and bursa of fabricious were harvested at 2 dpi, and the expression of *TRIM25* gene were measured by real-time PCR, ** $P < 0.01$ indicates the significant difference comparing NDV-infected chickens with uninfected chickens at 2 dpi.

CEFs significantly increased with increasing concentrations of dsRNA-mimetic poly(I:C) from 0.25 to 1.0 $\mu\text{g}/\text{mL}$ ($F = 27.62$, $df_1 = 5$, $df_2 = 12$, $P < 0.05$). On the contrary, a relatively high concentration of poly(I:C) at 4.0 $\mu\text{g}/\text{mL}$ resulted in a significant decrease in *TRIM25* expression. However, with increasing concentrations of dsDNA-analog poly(dA:dT), *TRIM25* expression levels showed an increasing trend in CEFs and peaked at transfection with 4 $\mu\text{g}/\text{mL}$ ($F = 84.07$, $df_1 = 5$, $df_2 = 12$, $P < 0.05$) (Fig. 7A). Meanwhile, *TRIM25* expression levels in CEFs transfected with 2 and 4 $\mu\text{g}/\text{mL}$ of poly(dA:dT) were significantly higher than that in CEFs transfected with others doses ($P < 0.05$).

We also assessed the expression levels of *TRIM25*, *IFN- α* , and *IFN- β* in CEFs after transfection with poly(I:C) and poly(dA:dT) at different timepoints, respectively. After transfection with 1.0 $\mu\text{g}/\text{mL}$ of poly(I:C), the expression levels of *TRIM25* ($F = 32.41$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$), *IFN- α* ($F = 28.50$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$) and *IFN- β* ($F = 615.72$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$) in CEFs peaked at 3 h and then gradually decreased, and were significantly lower at 6 and 9 h post-transfection than at 3 h post-transfection ($P < 0.05$) (Fig. 6B–D), respectively. Furthermore, expression levels of *TRIM25*, *IFN- α* , and *IFN- β* in CEFs at 3, 6, and 9 h post-transfection were significantly higher than in controls ($P < 0.05$), respectively. After transfection with 1.0 $\mu\text{g}/\text{mL}$ of poly(dA:dT), expression levels of *TRIM25* ($F = 199.3$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$), *IFN- α* ($F = 54.61$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$) and *IFN- β* ($F = 78.35$, $df_1 = 3$, $df_2 = 8$, $P < 0.05$) in CEFs gradually increased from 3 to 9 h post-transfection and peaked at 9 h to levels significantly higher compared to the other two timepoints ($P < 0.05$) (Fig. 7B–D). Meanwhile, expression levels of *TRIM25*, *IFN- α* ,

and *IFN- β* in CEFs at 3, 6, and 9 h post-transfection were also significantly higher than those in the control ($P < 0.05$). Overall, these results suggest that *TRIM25* might participate in viral dsRNA and dsDNA-triggered the antiviral immune response in chicken cells.

4. Discussion

In this study, we cloned and sequenced full-length chicken *TRIM25* cDNA, which contains a 1902-bp ORF, a 25-bp 5'-UTR and a 779-bp 3'-UTR. However, the cloned *TRIM25* cDNA (accession no.: KM879873) was shorter than the predicted *TRIM25* cDNA of *G. gallus* (accession no.: XM_415653.4) listed in the GenBank database. The predicted *G. gallus* *TRIM25* cDNA had a 2019-bp ORF without a 5'-UTR, and encoded a 672-amino acid protein. Intriguingly, the initial 92 bp of the 5'-terminus of the predicted *G. gallus* *TRIM25* cDNA was absent in our sequence, leading to a probable 633-amino acid protein encoded by the cloned *TRIM25* cDNA. Because our *TRIM25* sequence was cloned from chicken spleen, further studies are required to investigate whether the predicted *G. gallus* *TRIM25* mRNA is present in other tissues of chicken, and whether these two transcript variants of *TRIM25* are the result of 5'-alternative splicing of *TRIM25*.

The predicted chicken *TRIM25* protein had an RBCC motif at its N-terminal region and a B30.2 domain at its C-terminal region. The RBCC motif of chicken *TRIM25* consisted of a RING finger domain, a B-box1, a B-box2, and a coiled-coil region (Fig. 2). The RING finger domain of chicken *TRIM25* had the consensus C3HC4 structure that was previously identified as the characteristic signature motif of E3 ubiquitin ligases

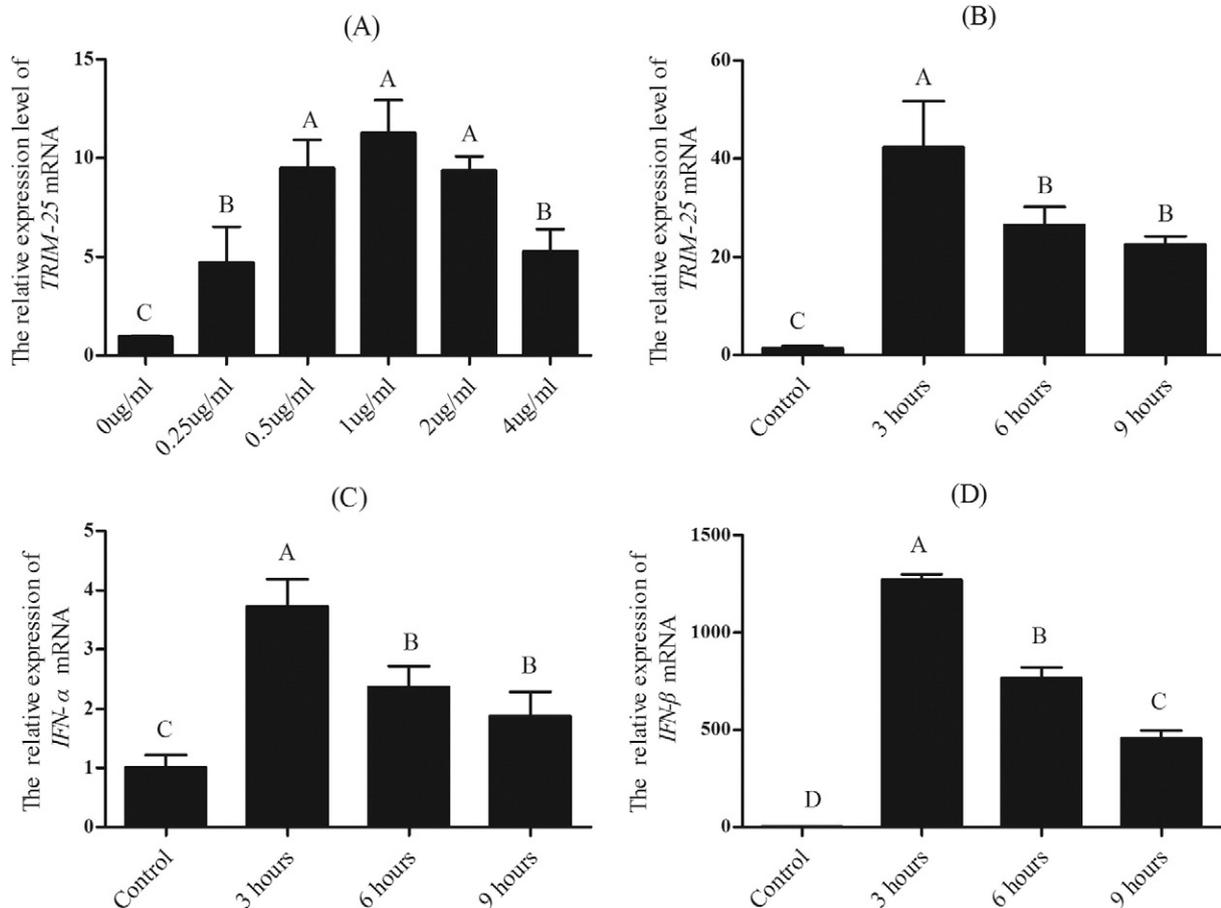


Fig. 6. The expression levels of *TRIM25*, *IFN- α* and *IFN- β* genes in CEF after transfection with poly(I:C). (A) CEF were transfected with different doses of poly(I:C) for 9 h and then analyzed for the expression levels of *TRIM25* gene by real-time PCR. After transfection with the poly(I:C), the expression levels of *TRIM25* (B), *IFN- α* (C) and *IFN- β* (C) genes in CEF were analyzed at 3, 6, 9 h, respectively. Data represent fold increases relative to cell transfected without poly(I:C). All data shown are the mean \pm SD and subjected into one-way ANOVA to identify differences between groups. Columns sharing different letters show significant difference ($P < 0.05$).

and reported to be crucial for the functioning of TRIM25 (Joazeiro and Weissman, 2000; Meroni and Diez-Roux, 2005; Gack et al., 2007). The B30.2 (PRY/SPRY) domain, which is composed of the PRY and SPRY subdomains, was first identified in the human class I major histocompatibility complex (Vernet et al., 1993). Analysis of its crystal structure revealed that PRY/SPRY forms a dimer, where an acceptor molecule binds to the concave surface of a donor sequence to form a putative interaction site. The formation of this dimer is also responsible for the interaction of TRIM25 with the N-terminal CARD domain of RIG-I in mouse (Grutter et al., 2006; Woo et al., 2006; Gack et al., 2007; James et al., 2007). The RING finger and B30.2 domains of chicken TRIM25 share high sequence similarity with human and murine TRIM25, indicating that chicken TRIM25 may have the same function as its human and murine orthologs.

Expression patterns of both human and murine TRIM25 in different tissues have been investigated previously (Inoue et al., 1993; Orimo et al., 1995; Inoue et al., 1999). Because of the presence of estrogen-responsive elements and IFN-stimulated response elements, TRIM25 is predominantly expressed in estrogen-targeting tissues, including the mammary glands, placenta, and uterus. Additionally, it is highly expressed in immune-related tissues such as the spleen, lungs, and thymus (Orimo et al., 1995; Inoue et al., 1999; Shimada et al., 2004). In the present study, we showed that TRIM25 was also dramatically expressed in the chicken spleen, thymus, and lungs, suggesting that TRIM25 might play an important role in innate immune response in chickens.

NDV is a negative-strand RNA virus that causes significant economic losses to the poultry industry (Kapczynski et al., 2013). NDV is mainly recognized by RIG-I in human and murine cells, and TRIM25 functions as an important E3 ubiquitin ligase for the activation of RIG-I and the subsequent signaling transduction that leads to the production of IFNs (Kato et al., 2006; Gack et al., 2007, 2009). Although RIG-I is absent in chickens (Barber et al., 2010), the expression of type I IFN, cytokines, and several IFN-stimulated genes is induced in the spleen, macrophages, embryo fibroblasts, and splenic leukocytes of chickens infected with NDV (Munir et al., 2005; Ahmed et al., 2007; Rue et al., 2011), indicating the presence of an unknown viral sensor that compensates for the absence of RIG-I. In this study, significantly increased TRIM25 expression was observed both in vitro and in vivo upon infection with NDV, suggesting a potentially antiviral role for TRIM25 in chickens. Due to the absence of RIG-I in chickens, the definite role of TRIM25 in the immune response to NDV is unclear. Recently, an increasing number of TRIM proteins have been found to be involved in modulating antiviral signaling pathways in mammals (Ozato et al., 2008; Kawai and Akira, 2011). For example, TRIM23-mediated interaction of the Lys27-linked polyubiquitin moiety with NF- κ B essential modulator is essential for virus infection-triggered nuclear factor kappa B (NF- κ B) and interferon regulator factor-3 (IRF3) activation (Arimoto et al., 2010). In contrast, TRIM21 negatively regulates the production of IFNs by interacting with both IRF3 and IRF7, thereby marking them for ubiquitination and degradation (Higgs et al., 2008, 2010). In addition, TRIM27 interacts with the IKK family of kinases and negatively regulates both NF- κ B-

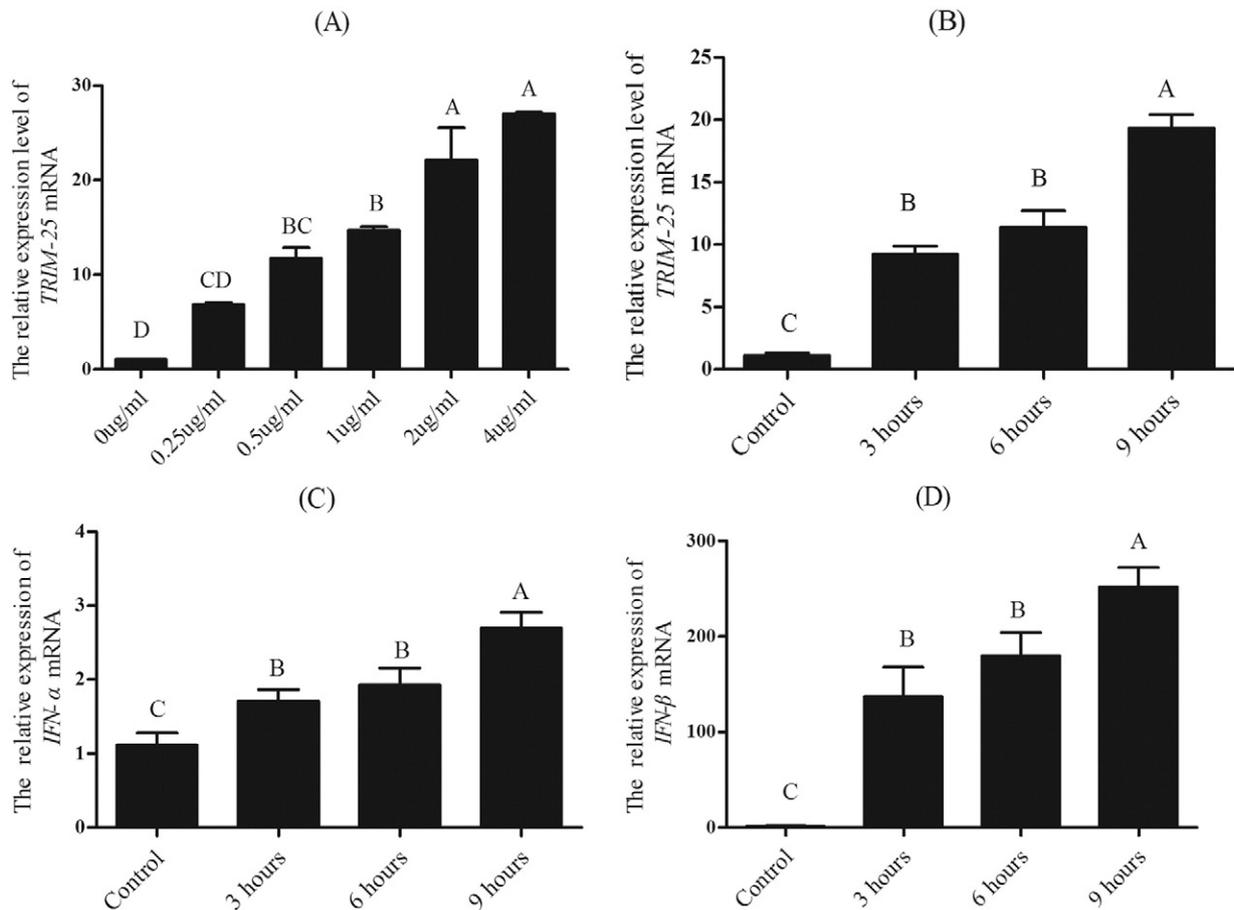


Fig. 7. The expression levels of TRIM25, IFN- α and IFN- β genes in CEF after transfection with poly(dA:dT). (A) CEF were transfected with different doses of poly(dA:dT) for 9 h and then analyzed for the expression level of TRIM25 gene by real-time PCR. After transfection with the poly(dA:dT), the expression levels of TRIM25(B), IFN- α (C) and IFN- β (D) genes in CEF were analyzed at 3, 6, 9 h, respectively. Data represent fold increases relative to cell transfected without poly(dA:dT). All data shown are the mean \pm SD and subjected into one-way ANOVA to identify differences between groups. Columns sharing different letters show significant difference ($P < 0.05$).

mediated and IRF-mediated gene expression (Zha et al., 2006). Taken together, these results suggest that TRIM25 might function as a crucial protein in the detection of NDV or in modulating NDV-triggered antiviral signaling pathways in chickens.

The innate immune system is the first line of host defense against invading pathogens (Barbalat et al., 2011). Host cells express pattern-recognition receptors that detect pathogen-associated molecular patterns (PAMPs) and trigger the production of type I IFNs (Pichlmair and Reis e Sousa, 2007; Takeuchi and Akira, 2010). Poly(I:C) and poly(dA:dT) are synthetic analogs of viral nucleic acids that function as PAMPs and trigger antiviral signaling pathways in human and murine cells (Gitlin et al., 2006; Kato et al., 2006; Ablasser et al., 2009; Chiu et al., 2009). Poly(I:C) induces *IFN- β* expression and is predominantly recognized by melanoma differentiation-associated protein 5 (MDA-5) in chicken cells (Karpala et al., 2011; Liniger et al., 2014; Lee et al., 2012; Hayashi et al., 2014). In agreement with the findings of previous studies, significant increases in the expression levels of chicken *IFN- α* and *IFN- β* were also observed in CEFs transfected with poly(I:C). Additionally, we observed that *TRIM25* expression levels were significantly upregulated in CEFs transfected with poly(I:C), indicating the potential role of *TRIM25* in dsRNA-triggered antiviral signaling pathways in chicken cells. Recently, it was reported that *TRIM25* not only activates the K63-polyubiquitination of the RIG-I signaling pathway (Gack et al., 2007), but also targets MAVS for ubiquitination and degradation after activating RIG-I and MDA-5, leading to the production of type I IFN (Castanier et al., 2012). Combined with the results of our study, this suggests that *TRIM25* might also catalyze ubiquitination of MAVS and induce the expression of *IFN- α* and *IFN- β* in chicken cells, after the recognition of dsRNA by MDA-5. Furthermore, *IFN- α* and *IFN- β* expression was induced in CEFs transfected with poly(dA:dT). These results suggest the existence of potentially unknown DNA sensors in chickens that recognize poly(dA:dT) motifs. The up-regulation of *TRIM25* expression in chicken cells transfected with poly(dA:dT) indicates the involvement of *TRIM25* in the cells' response to ectogenic DNA, but the underlying mechanism is unclear. Recently, it was reported that *TRIM56* and *RNF5* deliver K63- and K48-linked polyubiquitination moieties that positively and negatively modulate the poly(dA:dT) recognition pathways in human cells (Zhong et al., 2009; Tsuchida et al., 2010). This indicates that *TRIM25* also likely functions as an E3 ubiquitin ligase to modify dsDNA-induced antiviral signaling pathways in chicken cells. Moreover, in contrast to poly(I:C), the expression levels of *TRIM25* and *IFN- α/β* showed a gradual increasing trend in chicken cells transfected with poly(dA:dT), suggesting that different antiviral pathways are employed by the cells in response to infection with RNA and DNA viruses. Further studies are needed to elucidate the molecular mechanisms by which chicken cells distinguish between RNA and DNA viruses.

5. Conclusion

In this study, the molecular cloning, nucleotide sequencing, structural, and phylogenetic analyses of the chicken *TRIM25* gene were described for the first time. The ORF of chicken *TRIM25* gene consisted of 1902 bp that probably encoded 633 amino acid residues. Structural analysis indicated that RING finger and B30.2 (SPY/SRPY) domains might play an important role for the function of chicken *TRIM25*. qPCR results showed that the chicken *TRIM25* gene had high expression level in immune tissues. Furthermore, this is the first study to observe the increase in expression levels of the chicken *TRIM25* gene both in vitro and in vivo following infection with NDV, and in CEFs transfected with poly(I:C) and poly(dA:dT), respectively. Although the underlying mechanism is unknown, it is strongly indicated that chicken *TRIM25* might be an important component for the antiviral pathway in chickens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2015.02.025>.

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