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Chicken optineurin suppresses MDA5-mediated interferon β production

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ABSTRACT Chicken MDA5 (**chMDA5**), the essential accepted pattern recognition receptors for detecting cytoplasmic viral RNA in chicken, initiates interferon β (**IFN-β**) generation. However, there is an incomplete elucidation of regulating chMDA5-mediated IFN-β production. NEMO-related protein, optineurin, was identified as inhibitors of virus triggered IFN-β induction in human or mice. In this study, full length of chicken optineurin (**chOPTN**) was cloned from chicken embryo fibroblast, and its role in inhibiting IFN-β signaling pathway was further explored. Full-length chOPTN encodes 547 amino acids residues and contains unique LC3 interaction region and ubiquitin binding domain. Chicken optineurin mRNA and protein are widely expressed in different tissues, especially the heart, kidney, and bursal fabricius (**BF**). Overexpressed chOPTN not only inhibits poly I:C or homos-induced human IFN-β promoter activation in 293T cells but also suppresses poly I:C, infectious bursal disease virus (**IBDV**) genome double-strand RNA (**dsRNA**), and chMDA5-induced chicken IFN-β (**chIFN-β**) promoter activation. In addition, we first revealed that chOPTN negatively regulates chIFN-β production via inhibiting ubiquitination of chicken TBK1, which is dependent on the ubiquitin-binding domain of chOPTN. Moreover, chIFN-β stimulus, poly I:C, and IBDV genome dsRNA improve chOPTN expression. Endogenous chOPTN expression is also upregulated by IBDV infection in 293T, DF-1 cells, as well as in BF. Therefore, our results suggested that chOPTN plays an inhibition role of chMDA5-mediated chIFN-β signaling pathway in chicken cells.

Key words: chicken interferon β, chicken optineurin, inhibition, chicken MDA5

INTRODUCTION

The innate immune response is a highly conserved line of defense against invading pathogens, and the antiviral innate immune responses are induced by pattern recognition receptors with detection of specific molecular pattern's (Kato et al., 2006; Loo and Gale, 2011). The different classes of pattern recognition receptors implicated in the detection of virus contain endosomal toll-like receptors, cytosolic DexD/H-box retinotic acid inducible gene-I (**RIG-I**)–like helicases, and cytosolic DNA receptors (Bowie and Unterholzner, 2008; Broz and Monack, 2013). Recognition of double-stranded viral RNA by RIG-I allows its interaction with the mitochondrial adaptor protein MAVS (Seth et al., 2005). The recruitment of adaptor molecules leads to the activation of associated kinases and transcription factors, such as interferon regulatory factor 3 and 7 (**IRF3** and **IRF7**) and nuclear factor-κB, resulting in supporting type I interferon production and promoting host to restrict virus infection (Sun et al., 2006).

TBK1 is a well-investigated serine/threonine kinase functioning as an essential protein in several cell signaling pathways including autophagy-associated inhibition of pathogens infection and innate immune response (Pourcelot et al., 2016; Richter et al., 2016).

TBK1 contains a kinase domain, an ubiquitin-like domain, a dimerization domain, and a C-terminal adaptor-binding motif. Activation of TBK1 is essential for promoting type I interferon generation (Shu et al., 2013). As we all know, the activity of TBK1 is widely regulated by post-translational modification including ubiquitination and SUMOylation (Wang et al., 2014; Saul et al., 2015). Recently, TBK1 K63-linked poly-ubiquitination was shown to be important for lipopoly-saccharide- or RIG-I like receptor-induced IFN generation. The E3-ubiquitin ligase RNF128 was induced to facilitate K63-linked ubiquitination of TBK1 on multiple lysine residues (Song et al., 2016). Oppositely, several deubiquitinase removes K63-linked polyubiquitination to negatively regulate TBK1-supported pathway, such as deubiquitinating enzyme cylindromatosis and ubiquitin-specific protease 38 (Ahmed et al., 2011; Lin et al., 2016).

Optineurin, also called NRP (NEMO-related protein) or FIP-2, contains 53% of sequence similarity with NEMO (Slowicka et al., 2016). In human beings, optineurin has been linked to different pathologies, such as amyotrophic lateral sclerosis, because of mutation of essential residues of optineurin (Maruyama et al., 2010). To this moment, optineurin has been reported that it was involved in at least 4 critical unrelated functions: autophagy, antiviral immunity, membrane trafficking, and mitosis regulation (Slowicka et al., 2016). The relationship between human/mouse optineurin and TBK1 has been shown in such previous reports. Their interaction was critical for optineurin terminating RIG-I–mediated type-I interferon activation (Mankouri et al., 2010).

Infectious bursal disease virus (**IBDV**), one of nonenveloped virus of the *Birnaviridae* family, damages the immature B cells in the bursal of Fabricius of young chickens, leading to an immunosuppression and an increased rate of secondary infections (Sanchez and Rodriguez, 1999). The genome of IBDV, belonging double-strand RNA (**dsRNA**) family, contains 2 segments, segment A and segment B. Segment A contains 2 overlapping open reading frame (**ORF**), ORF1 and ORF2. Open reading frame 1 encodes viral nonstructural protein VP5 that is not essential for viral replication (Li et al., 2013). Open reading frame 2 encodes a large precursor polyprotein pVP2-VP4-VP3, which is auto-cleaved into separate VP2, VP4, and VP3 (Mata et al., 2018). VP2 is the major viral inner structural protein (Bloyet et al., 2016). VP3 is the multifunctional protein that includes viral replication complex assemble and viral capsid assemble (Liu et al., 2020). Segment B contains only 1 ORF that encodes the RNA-dependent RNA polymerase of IBDV (von Einem et al., 2004).

However, until now, there was no publications about function of chicken optineurin (**chOPTN**) involved in regulating innate immune responses and eventually regulating RNA virus replication. In this study, we first cloned the full-length cDNA of chicken optineurin and analyzed the protein's role in regulating type I interferon signaling. Our results revealed that chicken optineurin functions as an suppressor in chicken MDA5 (**chMDA5**)–mediated type I interferon production via inhibiting ubiquitination of chTBK1. We further found that dsRNA analog poly I:C, IBDV genome dsRNA, and even IBDV infection induces endogenous chicken optineurin expression, indicating that chicken optineurin may be essential for virus replication.

MATERIALS AND METHODS

Cell Lines and Virus

HEK293 T cells (ATCC CRL-11268) and the chicken fibroblast cell line DF-1 (ATCC CRL-12203) were generally maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (CCS30010.02; MRC; Australia), and IBDV virus JX-1 strain was sustained in our laboratory.

Antibodies and Reagents

Mouse anti-Flag and anti-HA monoclonal antibody were both purchased from Sigma-Aldrich (H3663 and F1804, St. Louis, MO). Rabbit anti-Myc and anti-β-actin antibodies were both obtained from Huaan Biological Technology (Hangzhou; China). The mouse Anti-Flag M2 Affinity Gel was purchased from Sigma-Aldrich (A2220). The mouse antioptineurin monoclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX; sc-166576). Mouse anti-VP3 and anti-VP4 polyclonal antibodies were both generated by immunization with prokaryotic purified protein and sustained in our laboratory. Horseradish peroxidase–labeled anti-mouse or anti-rabbit IgG were purchased from KPL (Milford, MA). The dsRNA analog poly I:C were purchased from InvivoGene (San Diego, CA). Mitogenic phorbol ester phorbol 12-myristate 13-acetate (**PMA**) and NP-40 lysis buffer were both purchased from Beyotime (S1819 and P0013 F, Shanghai, China).

Chicken Optineurin Cloning, Sequence Alignment, and Homology Analysis

Based on the chicken genomic sequence and National Center for Biotechnology Information published sequence of chicken optineurin (GenBank accession number NM_204236.1), gene-specific primers (Table 1) for PCR amplification of the complete coding region were designed using the Primer Premier 5 software. The software package DNAMAN 8.0 was used to deduce the amino acids sequence and to construct multiple sequence alignments of the amino acids sequences of optineurin proteins as well as to build the phylogenetic tree of optineurin. The protein accession numbers used in this comparison are *Homo sapiens* (NP_068815.2), *Sus scrofa* (NP_999126.1), *Bos taurus* (NP_001029774.1), *Gallus gallus* (NP_989567.1), and *Anas platyrhynchos* (XP_005018895.2).

Table 1. Primers used in this study.

Specific primers	Primers sequence (5′-3′)	Size
ChOPTN-F	GAATTCGCATGTCCAGCAAGCCGCAGATCC	
ChOPTN-R	GCCTCGAGTCAATTAATGCAGTCCATAACA	1,671 bp
ChTBK1-F	GAATTCGCATGCAGAGCACCTCGAATTACC	
ChTBK1-R	GCCTCGAGTTAGATACAGTCCACATTCCTC	2,190 bp
ChIRF3-F	GAATTCGATGGCAGCACTGGACAGCGAG	
ChIRF3-R	GGTACCTCAGTCTGTCTGCATGTGGTATTG	1,476 bp
qPCR ChOPTN-F	AATGGAGAACGGAATGGACAG	
qPCR ChOPTN-R	GTCCTTCATTGCTTGGTTGTG	145 bp
qPCR hOPTN-F	AGTGCTGAAGGAACTGAGTG	
qPCR hOPTN-R	TAAACTTCCATCTGAGCCCTG	150 bp
qPCR Chgapdh-F	TCTCTGGCAAAGTCCAAGTG	
qPCR Chgapdh-R	TCACAAGTTTCCCGTTCTCAG	135 bp
qPCR hgapdh-F	AATCCCATCACCATCTTCCAG	
qPCR hgapdh-R	AAATGAGCCCCAGCCTTC	122 bp

DNA Construction, Transfection, and Dual Luciferase Reporter Assays

The homos luciferase reporter plasmids hIFN-β-Luci, NF-κB-Luci, and Flag-hRIG-I (homos RIG-I) were kind gift from Ph.D Zhen Ding. The chicken luciferase reporter plasmid chIFN-β-Luci were sustained in our laboratory. Flag-chMDA5 and Flag-chMAVS were all all amplified by PCR from chicken embryo fibroblast and subsequently to be sub-cloned into Flag tag expression vector. Code sequences of chicken TBK1, IRF3, and optineurin were all amplified by PCR from chicken embryo fibroblast and subsequently to be subcloned into Flag or Myc tag expression vector. The mutations of chicken optineurin were constructed by site mutagens assays using the wild-type (**WT**) plasmid as template. All constructions of DNA were transfected into cells using the ExFect Transfection Reagent (T101-01/02; Vazyme Biotechnology, Nanjing, China) according to the manufacturer's descriptions. Dual luciferase assays were performed based on the manufacturer's instructions (DL101-01; Vazyme Biotechnology). Briefly, cells were transfected with the expression plasmid or the empty vector together with indicated reporter plasmid and pRL-TK plasmid expressing Renilla luciferase (0.1 μg/well; Promega) as the internal reference. All reporter assays were repeated at least 3 times.

qRT-PCR

Total RNA of the samples was prepared with the TRIzol reagent (Thermo Fisher, Waltham, MA) based on the manufacturer's descriptions. Reverse transcription of 1 μg total RNA was performed by using RevertAid RT Reverse Transcription Kits (Thermo Fisher, K1691) according to the manufacturer's protocol. The amplification of target gene was used to determine the transcript abundance and the transcripts of *gapdh* were used as the internal control. The relative abundance of transcripts was assessed using the ChamQTM Universal SYBR qPCR Master Mix (Q711-02/03, Vazyme Biotechnology) in ABI7500 sequence detector system (Applied Biosystems, Carlsbad, CA). The primers for qRT-PCR are available in Table 1.

Glutathione S-Transferase Pull-Down

Purified 10 μg glutathione S-transferase (**GST**) tag protein were either incubated with purified 10 μg His tag protein or whole cell lysates of transfected 293T cells. The mixtures were incubated on a rotator at 4°C for 2 h, and subsequently 100 μL of GST agarose (Thermo Fisher) was added for another 1 h at 4°C with agitation. The final mixture was then rinsed 5 times with lysis buffer to remove nonspecific binding. The precipitates were subjected to SDS-PAGE and processed for Western blot analysis.

Coimmunoprecipitation and Western Blotting

Coimmunopricipitation and Western blotting analyses were performed to confirm the interaction between chOPTN and chTBK1. HEK293 T cells seeded in 6-well plates were transfected with indicated plasmids for 48 h. The cells were lysed with NP-40 buffer for 30 min at 4°C. Protein samples were prepared as described above. Equivalent amounts of cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare, Chicago, IL). After blocking with 5% milk in phosphate-buffered saline containing 0.1% Tween 20 for 30 min at room temperature. The membranes were reacted with corresponding primary antibodies for 8 h at 4°C, followed by horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG at room temperature for 30 min. Finally, the blots membranes were subjected to develop with ECL detection reagents (E412-02, Vazyme Biotechnology) and scanned by ChemiDoc XRS + Imaging System (Bio-Rad, Hercules, CA).

Statistical Analysis

The statistical significance of differences between groups was determined using the Student t test. A

P-value of less than 0.05 was considered statistically significant.

RESULTS

ChOPTN Cloning and Sequence Analysis

Primers chOPTN-F and chOPTN-R (Table 1) were designed based on the sequence of chicken optineurin found in National Center for Biotechnology Information database and were used to amplify the potential chOPTN cDNA from chicken embryo fibroblast. We found that full length of chOPTN contains 1,670 bp and encodes 556 amino acids residues (Genbank accession number NP_989567.1; Figure 1A). A multiple sequence alignment suggested that the amino acids sequence of chOPTN is only 56% identical to that of *Homos sapiens*, *Sus scrofa,* and *Bos taurus* optineurin proteins. There is 87% identical shared between chicken optineurin and duck optineurin. Phylogenetic analysis showed that chicken optineurin and duck optineurin protein sequences were in the same subgroup (Figure 1B).

Previous reports indicated that optineurin, as a autophagy receptor, facilitates mitophagy via interacting with LC3B, because optineurin contains a classic LC3B interaction region (**LIR**), F*XX*I (Richter et al., 2016). Multiple sequence alignment showed that LIR of optineurin, FVEI, completely conserves in different species optineurin (Figure 1A). GST pull-down assays showed that chicken optineurin also interacts with chicken LC3B dependents, the residues of isoleucine 173 (I173), 1 essential amino acids of LIR (Figure 1C). Until now, there are no available reported studies on the issue distribution of chOPTN. So, we analyzed the expression profiles of chOPTN mRNA transcription level and revealed that chOPTN is widely expressed in different tissues, including heart, liver, spleen, lung, kidney, thymus, and bursal fabricius (**BF**), especially highly expressed in BF (Figure 1D). These results indicated that chOPTN expression is
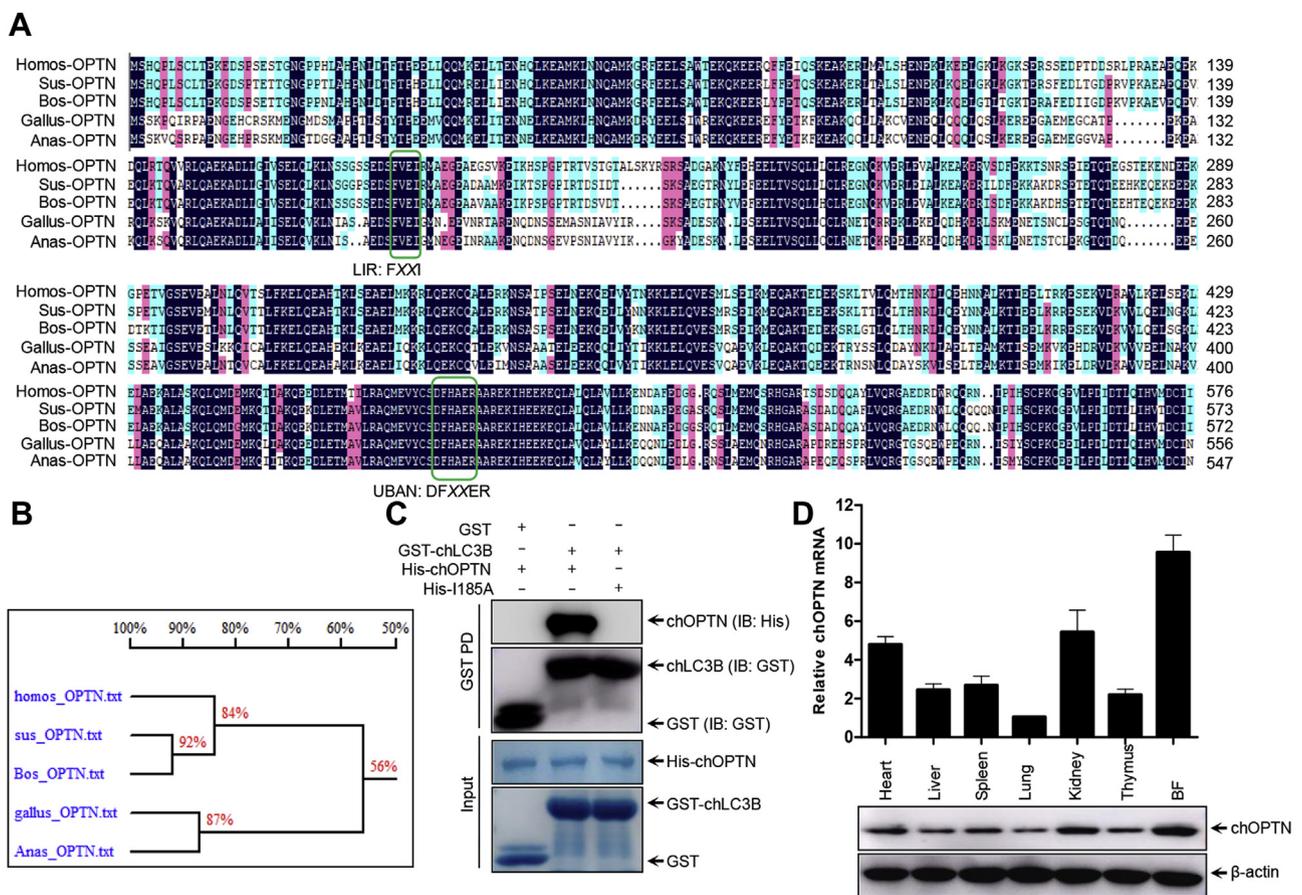

Figure 1. ChOPTN cloning and sequence analysis. (A) Amino acid alignments of *Homo sapiens* (NP_068815.2), *Sus scrofa* (NP_999126.1), *Bos taurus* (NP_001029774.1), *Gallus gallus* (NP_989567.1), and *Anas platyrhynchos* (XP_005018895.2) optineurin was performed and edited with DNAMAN 8.0 program. LIR sequence in red box means LC3 Interaction Region, and UBAN sequence in another red box means ubiquitin Binding Domain. (B) Phylogenetic tree of amino acid sequences of optineurin from different species. The tree was constructed using DNAMAN 8.0 program based on the multiple sequence alignment result of Figure 1A. (C) The interaction of gLC3B with chOPTN or its mutant in vitro. Purified GST or GST-chLC3B protein were incubated with purified His-chOPTN for GST pull down assay. The precipitates were subjected to SDS-PAGE and Western blotting using indicated antibody. (D) The transcription levels of chOPTN mRNA in healthy Chicken tissues by performing qRT-PCR and Western blotting. ChOPTN mRNA levels were expressed as fold expressions which were calculated based on the levels of chOPTN mRNA in the lung. ChOPTN protein levels were analyzed by Western blotting using optineurin polyclonal antibody. The β-actin band was used as the loading control. Abbreviations: ChOPTN, chicken optineurin; GST, glutathione S-transferase; IBDV, infectious bursal disease virus; LIR, LC3B interaction region; UBAN, ubiquitin biding domain.

not only restricted to tissues of the immune system but also is expressed in nonimmune tissues, increasing the possibility that chOPTN has other unknown functions in addition to its immune regulating roles.

ChOPTN Negatively Regulates chMDA5–Mediated IFN-β Promoter Activation

To further investigate the role of chOPTN in regulating type I interferon signaling pathway, DF-1 cells were cotransfected with chOPTN expression plasmid and a chicken IFN-β gene promoter luciferase reporter plasmid. The transfected cells were treated with poly I:C, a dsRNA analog stimulating chMDA5–mediated IFN-β induction. We found that chOPTN negatively regulates poly I:C–induced chIFN-β promoter activation (Figure 2B). In addition, chOPTN also inhibits poly I:C–induced homos IFN-β (**hIFN-β**) promoter activation in 293T cells (Figure 2A). Furthermore, the results of Figure 2C and Figure 2D showed that chOPTN not only suppresses hRIG-I–induced hIFN-β promoter activation in 293T cells but also inhibits chMDA5 induced chIFN-β promoter activation in DF-1 cells, in a dose-dependent manner. Human OPTN (**hOPTN**) is identified as suppressor of TNFα-induced NF-κB activation. Our results indicated that chOPTN also inhibits TNFα-induced NF-κB activation in 293T cells (Figure 2F), and also hOPTN inhibits chMDA5–mediated interferonβ promoter activation in DF-1 cells (Figure 2G), raising the possibility that the sole function of chOPTN is not changed, although hOPTN and chOPTN are located in different subgroup (Figure 1B). Here, dual luciferase reporter assays revealed that chOPTN significantly blocks IBDV genome dsRNA–induced chIFN-β promoter activation (Figure 2E). To identify the step of the IFN pathway targeted by the inhibitory function of chOPTN, DF-1 cells were cotransfected chMDA5/chMAVS/chTBK1/chIRF3 with or without chOPTN together with chIFN-β-Luci plasmid for 36 h. Dual luciferase reporter assays showed that chMDA5/chMAVS/chTBK1/chIRF3 significantly improves chIFN-β promoter activation (Figure 2G). Chicken optineurin significantly inhibits chMDA5, chMAVS, and chTBK1, but not chIRF3-induced chIFN-β promoter activation. This result strongly suggested that chOPTN inhibitory function targets chTBK1 (Figure 2G). Taken together, chOPTN negatively affects chIFN-β promoter activation via targeting chTBK1 step.

ChOPTN Inhibits Ubiquitination of chTBK1

The ubiquitin biding domain (**UBAN**) of optineurin, DF*XX*ER, is highly conserved among different species optineurin (Figure 1A). This domain was reported to interact with WT ubiquitin, linear ubiquitin as well as K63-linked ubiquitin and to be critical for initiating mitophagy and inhibiting NF-κB signaling pathway

(Slowicka et al., 2016). Purified His-Ub, His-K63, and His-48–linked ubiquitin protein along with GST-chOPTN were used for GST pull-down assay. The result of Figure 3A showed that chOPTN efficiently binds with both WT and K63-linked ubiquitin chain, but not K48-linked ubiquitin chain, but UBAN mutation of chOPTN, chOPTND453 N, impairs the interaction with ubiquitin chain (Figure 3A). Coimmunopricipitation assays revealed that chOPTN interacts with chTBK1 (Figure 3B). Further, GST pull-down assays showed that UBAN domain of chOPTN is essential for binding with chTBK1. Because TBK1 activity is regulated by its phosphorylation and ubiquitination, we hypothesized that optineurin could exert inhibitory effect on chTBK1 by suppressing chTBK1 ubiquitination, and subsequently, suppressing chIFN-β promoter activation. Ubiquitination assays showed that ubiquitination of chTBK1 is remarkably inhibited by WT chOPTN but not chOPTND453 N (Figure 3D). In addition, chOPTND453 N could not block chMDA5-induced chIFN-β promoter activation (Figure 3E), indicating that ubiquitin binding domain of chOPTN is critical for binding with chTBK1 and thereby negatively regulates chIFN-β production. Altogether, we conclude that ubiquitin binding domain of chOPTN is essential for inhibiting chIFN-β production.

IBDV Infection Induces chOPTN Expression

To investigate whether the expression of chOPTN was regulated by RNA virus infection or by poly I:C stimulation, DF-1 cells were treated with poly I:C or infected with IBDV; then, the total RNAs were extracted, and the transcriptional level of chOPTN was monitored by qRT-PCR. Meanwhile, total cellular protein were subjected to Western blotting using optineurin antibody. As shown in Figure 4A, both mRNA and protein level of chOPTN was upregulated by poly I:C. As positive control, mitogenic phorbol ester PMA was reported to induce hOPTN expression (Genin et al., 2015). Expression of chOPTN is also induced by PMA in DF-1 cells (Figure 4B). In addition, IBDV genome dsRNA could also significantly improve the expression of chOPTN (Figure 4C). To further explore whether optineurin expression is induced by RNA virus infection, 293T and DF-1 cells were infected with 1 multiplicity of infection of IBDV for different time. Western blotting assays showed that both mRNA and protein level of chOPTN are significantly improved by IBDV infection (Figures 4D, 4E). In addition, BF infected with IBDV for 60 h and 72 h were subjected to western blotting. As shown in Figure 4F, IBDV infection significantly induce the expression of chOPTN in vivo. These results indicated that chMDA5 ligands, poly I:C, or IBDV genome dsRNA, and even RNA virus infection could improve the endogenous chOPTN expression, which may be functioned as negative feedback regulating type I interferon production, and eventually regulates virus replication.
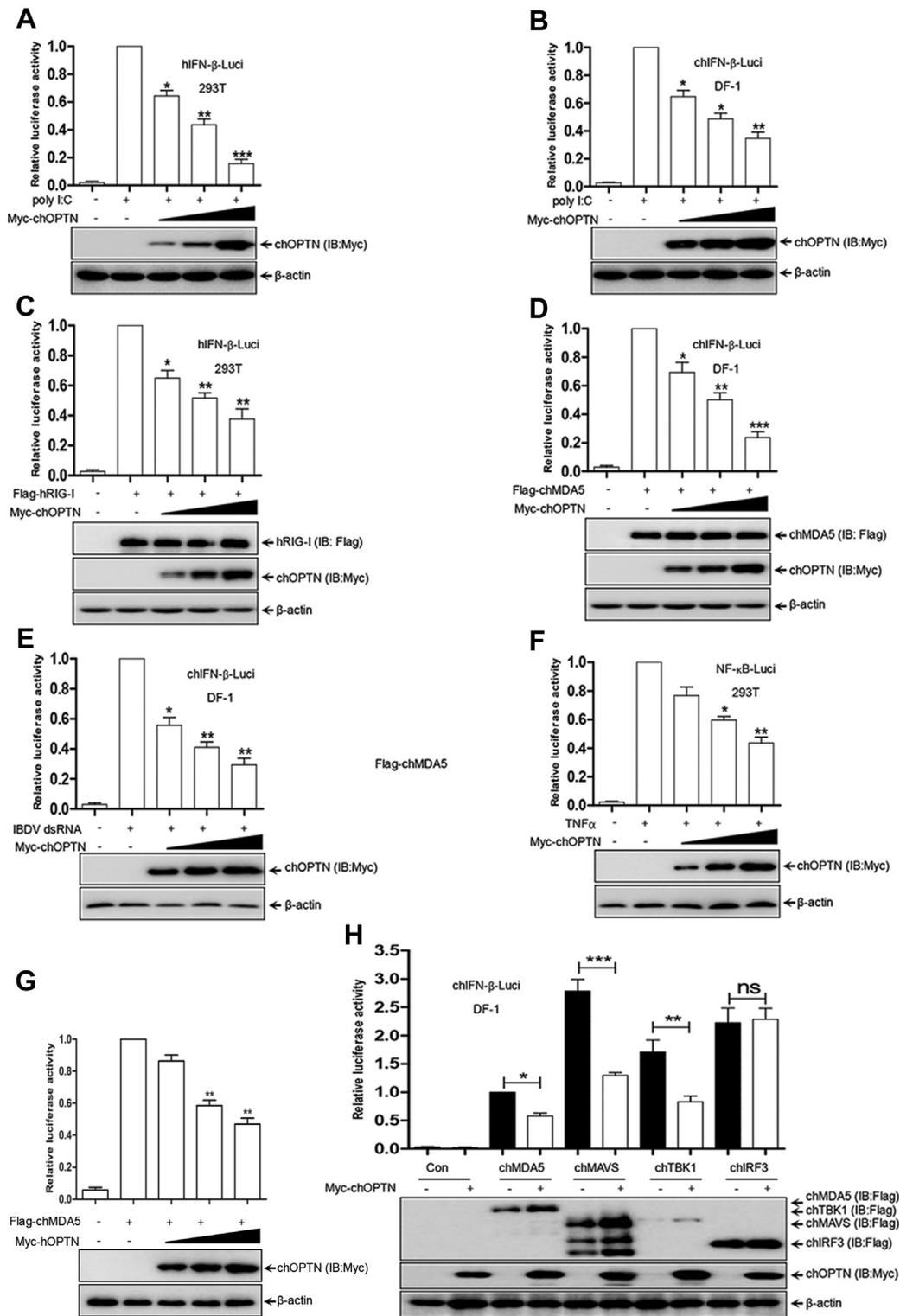
Figure 2. The overexpression of chOPTN attenuated the chIFN-β promoter activation. (A and B) ChOPTN inhibits hIFN-β promoter activation (A) or chIFN-β promoter activation induced by ploy I:C. 293T cells were transfected with indicated plasmids for 24 h. The transfected cells were treated with 100 ng poly I:C for another 18 h. (C and D) ChOPTN suppresses hIFN-β promoter activation in induced by hRIG-I (C) or chIFN-β promoter activation induced by chMDA5 (D). 293T cells (C) or DF-1 cells (D) were transfected with indicated plasmids for 24 h. (E) ChOPTN inhibits chIFN-β promoter activation induced by IBDV genome dsRNA. DF-1 cells were transfected with indicated plasmids for 24 h. The transfected cells were treated with 50 ng dsRNA for another 18 h. (F) ChOPTN inhibits hNF-κB promoter activation induced by TNF-α. 293T cells were transfected indicated plasmids for 24 h. The transfected cells were treated with 100 ng TNF-α for another 18 h. (G) hOPTN inhibits chIFN-β promoter activation induced by ChMDA5. DF-1 cells were transfected indicated plasmids for 24 h. (H) ChOPTN inhibits the step of chTBK1 mediated chIFN-β activation. DF-1 cells were transfected with indicated plasmids for 24 h. After that, collecting the cell lysates for dual Luciferase activity. The results are presented as relative firefly Luciferase activity. Data are means from 3 independent experiments. Mean ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: ChOPTN, chicken optineurin; hIFN-β, homos IFN-β; IBDV, infectious bursal disease virus.
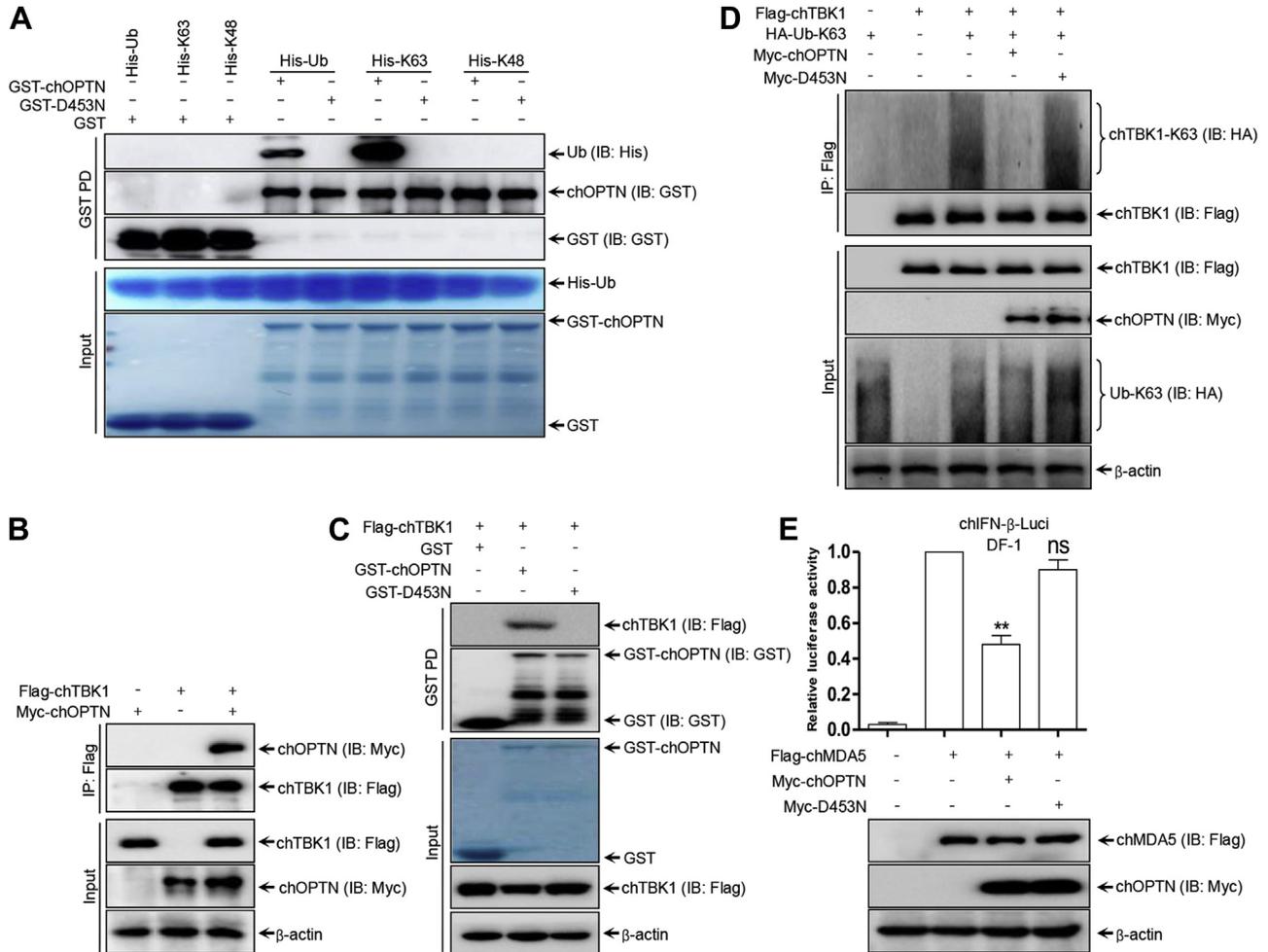
Figure 3. Chicken optineurin inhibits ubiquitination of chTBK1. (A) ChOPTN interacts with ubiquitin. Purified GST-chOPTN or its mutant's protein were incubated with either purified His-Ub, His-K63, or His-K48. The mixtures were then subjected to GST pull-down and subsequently to Western blotting with indicated antibody. The purified protein were determined by Coomassie blue staining. (B) ChOPTN interacts with chTBK1. 293T cells were transfected with indicated plasmids for 48 h. The lysates of transfected cells were subjected to Western blotting with indicated antibodies. (C) Asp 453 residues of chOPTN is required for binding with chTBK1. Purified chOPTN or its mutants protein were incubated with lysates of 293T cells transfected Flag-chTBK1 and then subjected to GST pull-down assay. The precipitates were analyzed by Western blotting with indicated antibody. The input purified GST-chOPTN protein were determined by Coomassie blue staining. β-actin band was detected as loading control. (D) ChOPTN inhibits ubiquitination of chTBK1. 293T cells were transfected indicated plasmids for 48 h. The transfected cells were lysed by NP40 buffer to subjected to ubiquitination assays. β-actin band was detected as loading control. (E) Asp 453 residues of chOPTN is required for inhibiting chIFN-β promoter activation. DF-1 cells were transfected with indicated plasmids for 24 h. The whole cell lysates were subjected to dual reporter assay. The results are presented as relative firefly Luciferase activity. Data are means from 3 independent experiments. Mean ± SD, **$P < 0.01$, Abbreviations: ChOPTN, chicken optineurin; GST, glutathione S-transferase; IBDV, infectious bursal disease virus; ns, nonspecific.

DISCUSSION

RIG-I like receptors receptors including MDA5 and RIG-I were reported as critical detector of RNA virus infection (Broquet et al., 2011). But, chicken RIG-I was not identified instead of chicken MDA5. Based on previous research, chicken MDA5 was identified as sensor of RNA viral genome to initiate chicken type I interferon production (Liniger et al., 2012). Chicken MAVS was further cloned to be as the adaptor of chicken MDA5 to support type I interferon generation (Cheng et al., 2017). chTBK1, a multiple function kinase, was reported to interact with chicken MAVS or STING to activate IRF3 to initiate transcription (Cheng et al., 2017). However, this regulation mechanism or molecular regulatory mechanism remains nuclear.

To better understand the biological role of chicken optineurin in chMDA5-mediated type I interferon production, we cloned the chOPTN from chicken cells that shared a high amino acid sequence similarity with duck optineurin but distinguished from mammalian optineurin (Figures 1A, 1B). Multiple sequence alignment revealed that chOPTN contains a highly conserved classical LIR, FVER. This region of chOPTN is responsible for interacting with LC3 (Figures 1A, 1C), suggesting that chOPTN may play an important role in autophagy or mitophagy. The constitutive mRNA transcription of the chOPTN gene was analyzed in all the selected tissues of healthy chicken (Figure 1D). Interestingly, chOPTN was found to be highly expressed in BF, the central immune organism of young chicken. This indicated a correlation between chOPTN and innate immunity.
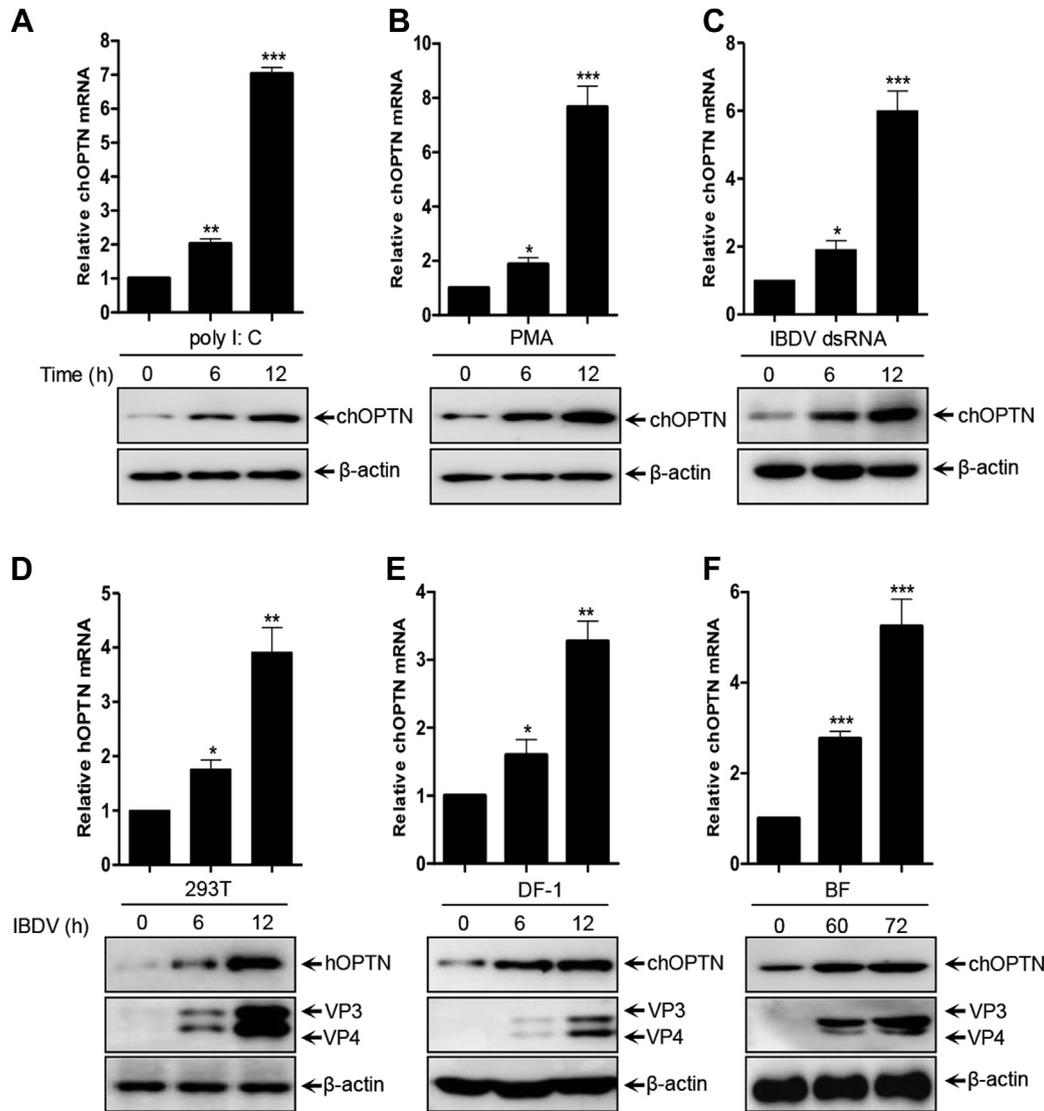
Figure 4. IBDV infection induces optineurin expression. (A, B, and C) poly I:C (A), PMA (B), IBDV genome dsRNA (C) supports endogenous optineurin expression. DF-1 cells were treated with poly I:C (A), PMA (B), and IBDV genome dsRNA (C) for different times. The total cellular RNA were extracted to perform qRT-PCR to assess optineurin mRNA level. In another, the cells were lysed with NP40 buffer to subjected to Western blotting with OPTN antibody. β-actin band was detected as loading control. (D, E, and F) IBDV infection promotes endogenous optineurin expression. 293T cells (D), DF-1 cells (E) were infected with 1 MOI IBDV for different times. Bursal Fabricius (BF) (F) derived from chicken infected with IBDV for different times. The total cellular RNA were extracted to perform qRT-PCR to assess optineurin mRNA level. In another, the cells were lysed with NP40 buffer to subjected to Western blotting with optineurin antibody. β-actin band was detected as loading control. Data are means from 3 independent experiments. Mean ± SD, *$P < 0.01$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: dsRNA, double-strand RNA; hOPTN, Human OPTN; IBDV, infectious bursal disease virus: MOI, multiplicity of infection; PMA, phorbol 12-myristate 13-acetate.

To further investigate the role of chOPTN in type I interferon generation, we performed the hOPTN or chOPTN overexpression experiment. We found that overexpression of chOPTN significantly inhibits poly I:C-induced IFN-β promoter activation (Figures 2A, 2B). In addition, both human and chicken IFN-β activation and was inhibited by chOPTN expression (Figures 2C, 2D). These further increased that possibility that the function of chOPTN and hOPTN in suppressing IFN-β generation is highly conserved in different species. We further found that chOPTN inhibits the chicken IFN-β production at the step of chTBK1 (Figure 2E).

The chTBK1 is required for chMDA5 activated IFN-β activation. The molecular regulation of chTBK1 is not

fully understood. Coimmunopricipitation experiment suggested that chOPTN is efficiently interacted with chTBK1. This interaction is dependent on the ubiquitin binding domain of chOPTN (Figures 3B, 3C). These domain is highly conserved in different species and required for binding with ubiquitin chain (Figures 1A, 3A). In vivo ubiquitin assay revealed that chOPTN inhibits the ubiquitination of chTBK1, which is dependents the D453 residues of chOPTN, the essential amino acid for binding with ubiquitin. Furthermore, D453 mutation of chOPTN did not inhibit IFN-β activation (Figure 3D).

Chicken MDA5 together with other immune molecules elicit type I interferon production and fight viral infection. On the hand, viruses develop multiple

strategies to evade the cascade of the innate immune system to facilitate their replication. Among of viral proteins of IBDV, the nonstructural protein VP4 is believed to be the important protein IFN inhibitor of IBDV (Li et al., 2013). However, whether chOPTN is the direct target of IBDV requires further explored.

In this study, we identified the chOPTN as a chTBK1 interactive protein and concluded that chOPTN is involved in blocking chMDA5 triggered type I IFN signaling in chicken cells. We also reported that the endogenous chOPTN expression was significantly induced by viral RNA analog, poly I:C, or IBDV genome dsRNA. In addition, IBDV infection also severely promotes endogenous chOPTN expression. This mechanism of a feedback regulation of innate immune system may be activated by IBDV or other viral infection and ultimately to promote viral replication. Hence, we guessed that VP3 or another viral protein of IBDV could interact with chOPTN to regulate cellular innate immune responses. Furthermore, this potential novel mechanism of IBDV suppressing innate immunity need to be further explored, leading us to extensively understand molecular features of IBDV, because IBDV is named as immunity suppression virus (Bottcher et al., 1997). Taken together, there result will further improve our understanding of the regulatory mechanism of chOPTN in type I interferon signaling in chicken cells.

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DISCLOSURES

All of the authors declare no conflict of interest.

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