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Chicken interferon-induced transmembrane protein 1 promotes replication of coronavirus infectious bronchitis virus in a cell-specific manner

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Abstract

Interferon-induced transmembrane proteins (IFITMs) are broad-spectrum antiviral proteins that inhibit numerous virus infections by impeding viral entry into target cells. However, increasing evidence suggests diverse functions of IFITMs in virus infection, especially with the coronavirus. We analyzed the effect of chicken interferon-induced transmembrane proteins (chIFITMs) on coronavirus infectious bronchitis virus (IBV) infection *in vitro*. We demonstrated that the antiviral effects of IFITMs are dependent on cell and virus types. The overexpression of chIFITM1 dramatically promoted the replication of IBV Beaudette strain in the chicken hepatocellular carcinoma cell line, LMH. Mechanistically, chIFITMs share roughly the same subcellular localization in different host cells, and overexpressed of chIFITM1 have no effect of viral attachment and entry. Further studies revealed that mutations of amino acids at key positions (60KSRD63, 68KDFV71) in the intracellular loop domain (CIL) caused loss of the promoted function. Interaction with downstream proteins in co-response to viral infection could be the primary reason behind variable functions of chIFITM1 in different cells. In all, our study explored the functions of chIFITMs in viral infection from a new perspective.

Keywords: IFITM, IBV, Chicken Virus, LMH, Coronavirus

1. Introduction

Interferon-induced transmembrane proteins (IFITMs) belong to a family of small transmembrane proteins. The IFITM gene family was first discovered in 1984 in neuroblastoma cells treated with interferon (IFN) [1]. Subsequent studies have demonstrated

that IFITMs are widespread in vertebrate cells. IFITMs are highly expressed following stimulation by IFNs and therefore are a class of interferon-stimulated genes (ISGs). However, further studies revealed that the expression of IFITMs can also be induced by other factors such as oncostatin M and IL-6 [2]. IFITM genes have certain orthologues in most vertebrate animals. For example, human IFITMs genes include *Ifitm1*, *Ifitm2*, *Ifitm3*, and *Ifitm5* which are clustered on chromosome 11 [3]. Similarly, mouse *Ifitm1*, *Ifitm2*, *Ifitm3*, *Ifitm5*, and *Ifitm6* are located on chromosome 7, and *Ifitm7* is located on chromosome 16. To date, five IFITM genes have been identified in chickens, including *Ifitm1*, *Ifitm2*, *Ifitm3*, *Ifitm5*, *Ifitm10*, and all of them are clustered on chromosome 5 [4; 5]. Among them, IFITM1, IFITM2, and IFITM3 have been extensively studied because of their antiviral function.

IFITMs are multifunctional proteins, with functions in diverse biological processes, including cancer development, germ cell homing and maturation, immune cell signaling, and bone mineralization [6; 7; 8]. One of their most well-known functions is their antiviral role in innate and adaptive immunity. Because IFITM proteins were identified as anti-IAV restriction factors in 2009 [9], numerous pathogenic viruses have been reported to be inhibited by IFITMs, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), dengue virus (DENV), Ebola virus (EBOV), influenza A virus (IAV), West Nile virus (WNV), severe acute respiratory syndrome coronavirus (SARS-CoV), and severe acute respiratory syndrome coronavirus (SARS-CoV-2) [10; 11; 12; 13]. However, studies on avian IFITMs are scarce. The IFITM1 and IFITM3 of chicken and duck are known to restrict influenza virus, rabies virus [4; 10], and avian tembusu virus (ATMUV) [14] infection *in vitro*. Transgenic chicks expressing *ifitm1* have been demonstrated to restrict H5N1 influenza viruses [15]. Recent studies have demonstrated that infection with IBV will increase the expression of IFITMs in chicks [16].

The mechanism underlying IFITM-mediated inhibition of viral infection remains elusive although interrupting the membrane fusion between viral envelope and cellular membranes has been considered the major molecular mechanism [17]. Certain non-enveloped viruses, such as reovirus [18] and foot-and-mouth disease virus [19], can be inhibited by IFITMs. In addition, certain viruses can hijack IFITMs to facilitate their infections, such as human coronavirus OC43 (HCoV-OC43) [20] and human cytomegalovirus (HCMV) [21]. A recent study proved that artificial overexpression of IFITMs blocked SARS-CoV-2 infection; however, endogenous IFITM expression results in efficient infection of human lung cells by SARS-CoV-2 [22]. Several studies have demonstrated that the antiviral potency of IFITM proteins varies among different cell types [21], suggesting that IFITMs can work with other cellular proteins to modulate viral infection [17].

In this study, we explored the functions of chicken IFITMs in the infection by coronavirus infectious bronchitis virus (IBV) *in vitro*. The result showed that chIFITMs have different functions in different host cells. In particular, chIFITM1 greatly promoted the replication of IBV in the chicken hepatocellular carcinoma cell line, LMH. We analyzed this

abnormal phenomenon by studying virus adsorption and entry into cells, subcellular localization, protein structure, and interacting protein network. These results enhance our understanding of chIFITMs and could be applied to control virus or vaccine production in the future.

2. Methods

2.1 Viruses and Cell Lines

The IBV Beaudette strain was kindly gifted by Prof. Ding-Xiang Liu, Nanyang Technological University. The IBV M41 strain, a wild-isolated IBV strain (GenBank: MT563407.1), Newcastle disease virus strain (NDV F48E9) and a wild-isolated FADV strain SCnj1601 (Genbank: KY927938) [23] were stored at the Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province and propagated in 10-day-old embryonated specific pathogen-free (SPF) eggs (Boehringer Ingelheim Vital Biotechnology Co. Ltd., Beijing, China) when used as previously described [24]. Viral titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) or median embryo lethal dose (EID_{50}). Specific details of virus infection on LMH, DF-1, HD11, and Vero have been previously described [25; 26]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gemini, USA) supplemented with 10% fetal bovine serum (FBS, Gemini, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin sulfate (Solabio, USA).

2.2 Plasmids

To express the chIFITM1/2/3 and human IFITM1 (huIFITM1) proteins, FLAG-tagged (DYKDDDDK) chIFITM1/2/3 and huIFITM1 genes (Gene ID: 422993, 107053353, 770612, 8519) were cloned into the pcDNA3.1 vector. Alanine scan mutants plasmids based on pcDNA3.1-chIFITM1 were constructed to verify the key functional regions of chIFITM1 using the QuickMutation™ Site-Directed Mutagenesis Kit (Beyotime, China). In addition, the chicken endosomal marker chRab5a (pEGFP-chRNA5a) was constructed to detect the intracellular location of chIFITMs. All recombinant plasmids were extracted using the GoldHi EndoFree Plasmid Maxi Kit (CWBIO, China). Recombinant plasmids were transfected into cells using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

2.3 DNA/RNA extraction and Quantitative Real-time PCR (qRT-PCR)

Viral DNA was extracted from infected cells using the TIANGEN Genomic DNAKi -t (Beijing, China). The total RNA was prepared from cells using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. One microgram of the total RNA was reverse transcribed using a Prime ScriptTMRT Reagent Kit (TaKaRa, Japan) to synth -esize cDNA. Next, qRT-PCR was performed to analysis the expression of correspondi -ng mRNAs (Primers IBV Beaudette forward: TGCTGCTAAGGGTGCTGATACT, reve -rse: AGGTCCGCCATCCGAGAATC; IBV WT forward: CGCTCAAGTTCAAGACCT GCTA, reverse: CATCATCCTGCTTCTTCGGCTT; IBV M41 forward: GTGGTCGCA

GGAGTGGTTCT, reverse: AGCCATTTCATCAGCCTTAGCC; NDV-F48E9 forward: A AGAAGCAAATCGCCCC, reverse: ACGCTTCCTAGGCAGAG; FADV forward: ACG TACATGGAGCACCCGG, reverse: CCTTGGGGAGGTCGGTTCTC). The Cha-mQ Uni -versal SYBR qPCR Master Mix was purchased from Vazyme (Nanjing, Chin-a). Rela -tive fold changes in gene expression were normalized against chGAPDH or huGAPD H using the $2^{-\Delta\Delta Ct}$ threshold method.

2.4 Indirect Immunofluorescence Assay and Confocal Microscopy

Indirect immunofluorescence assay (IFA) was used to observe the proliferation of the virus and the expression of proteins in the cells. Briefly, Cells infected with IBV or transfected with plasmids were fixed and incubated with mouse anti-IBV N protein monoclonal antibody (Novus, USA) or rabbit anti-flag monoclonal antibody (CST, USA) at 37°C for 2 h. Afterward, cells were incubated with the secondary antibody (Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) or Alexa Fluor 555-labeled donkey anti-rabbit IgG (H+L), Beyotime, China) at 37°C for 2 h, and stained with DAPI for 10 min before imaging under a fluorescence microscope (Leica DMi 8). The cells were cultured in 35 mm glass dishes and processed for IFA, followed by observation under a multi-scan using confocal microscopy (Zeiss Cell Observer SD).

2.5 Western blot analysis

The cells were washed with pre-cooled phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (Beyotime, China) on ice for 30 min, and centrifuged at 3000 rpm for 10 min. Afterwards, mix an appropriate amount of supernatant with the loading buffer in a boiling water bath for 15 min to denature the protein. After SDS gel electrophoresis, the protein samples were transferred to polyvinylidene fluoride (PVDF) membranes and blocked with QuickBlock™ Blocking Buffer (Beyotime). Next, the membranes were incubated with antibodies including rabbit anti-flag monoclonal antibody (CST, USA), anti-GAPDH (GAPDH mouse monoclonal antibody, Beyotime) overnight at 4℃, followed by incubation with the secondary antibody at room temperature for 2 h. Finally, the protein bands were imaged using BeyoECL Plus (Beyotime).

2.6 Virus attachment and entry assays

To determine whether the overexpression of IFITM1 affects the attachment and entry of IBV to LMH cells, 24 h after transfection, cells were inoculated with IBV (MOI = 10) at 4°C for 1 to 2 h to ensure the virus was completely attached to the cell receptor. Next, the cells were washed thrice with pre-cooled PBS to remove unbound viruses. Trizol was added to lyse the cells, followed by extraction of the total RNA. Or the cells were shifted to 37°C with 5% CO_2 to allow virus internalization, after washing the unbound virus with pre-cooled PBS. After 2 h, 1 mM proteinase K was added and cells were incubated for 30 s to wash away the virus particles bound to the receptor but that had not entered the cells. Lastly, the cells were washed with PBS 2 to 3 times, and Trizol was added to lyse the cells and extract the total RNA. The viral RNA in each treatment was detected by RT-qPCR.

2.7 Immunoprecipitation and LC-MS analysis

The chIFITM plasmids were transfected into HD11 cells or LMH cells seeded on six-well plates. After 24 h, the cells were washed with pre-cooled PBS, lysed with RIPA lysis buffer (Beyotime) on ice for 30 min, and centrifuged at 3000 rpm for 10 min. Then, the mixture was incubated with anti-Flag Affinity Gel (Beyotime) overnight at 4℃. After washing with TBS several times, the protein binding to IFITM1 was eluted with the eluent, and afterward, SDS-PAGE was performed, and the target band was cut. Liquid chromatography-mass spectrometry (LC-MS) sequencing was performed by Shanghai Luming Biological Co., Ltd. using Ultimate 3000 nano-based ultra-high Liquid chromatography and Q Exactive plus high-resolution mass spectrometer. The sequencing results were analyzed using Proteome Discover 2.5, and the chicken protein library (GRCg7b) was used as the reference protein library. Gene Ontology (GO) analysis, KEGG pathway analysis, and STRING (protein-protein interaction analysis) were performed to analyze the interaction proteins with chIFITM1 in different cells.

2.8 Statistical Analysis

All data were analyzed using the Prism 7 software (GradphPad Software, La Jolla, CA, USA), and Student's *t*-test was used for all statistical analyses. Data are presented as mean ± standard deviation (SD) of three independent experiments. *p*-Values < 0.05 were considered statistically significant.

3. Results

3.1 IBV Beaudette strain proliferates on LMH cells, whereas other wild strains cannot

Although IBV is the earliest isolated coronavirus, research on this virus is scarce due to the fact that almost all strains cannot adapt to passaged cells. To determine whether IBV proliferates on chicken LMH cells, LMH cells were inoculated with IBV Beaudette strain (Vero passaged), the M41 strain, a wild isolated strain (GenBank: MT563407.1), and negative control were used. All viruses were serially passaged thrice on LMH cells; however, a typical cytopathic effect (CPE) was only observed after the Beaudette strain infection. In addition, the Beaudette strain was serially propagated for 20 passages in LMH cells. To detect the replication status of IBV in each passage, the viral RNA was detected from 1, 3, 5, 10, 15, and 20 passages, and bright bands were observed using PCR and gel electrophoresis (Fig. 1A). Next, an indirect immunofluorescence assay (IFA) was used to observe the P20 infective process. Almost no fluorescence was observed in the negative control; however, strong fluorescence was observed in P20-infected LMH cells (Fig. 1B). In addition, the S1 gene of the P20 virus was sequenced and showed no base mutation (data not shown). These results indicated that IBV Beaudette strain efficiently and stably passaged on LMH cells.

3.2 chIFITM function is closely related to cell and virus types

The pcDNA3.1-chIFITM1/2/3 plasmid was transfected into three avian cell lines, namely, HD11, DF-1, and LMH, and subsequently infected with the IBV Beaudette strain.

Twenty-four hours after infection, viral mRNA, and viral N protein expression were calculated to assess the impact of overexpressing chIFITM1 on IBV infection. The results showed that chIFITM1/2/3 was correctly expressed in three cell lines, overexpression of IFITMs had no significant effect on cells viability (SUPPLEMENTARY MATERIAL 1). Compared with the negative control group (transfected with pcDAN3.1 plasmid), chIFITM1/2/3 effectively inhibited the replication of IBV in HD11 cells (Fig. 2A). However, in DF-1 cells, only chIFITM2 showed limited inhibition (Fig. 2B). Surprisingly, chIFITMs promoted IBV proliferation in LMH cells. In particular, the amount of IBV in chIFITM1-overexpressed LMH cells was hundreds of times higher than that in the control group, as evidenced by both mRNA and protein levels. What's more, distinct CPE was observed in a considerably shorter time in chIFITM1-overexpressed LMH cells compared to the negative control group (Fig. 2C).

The overexpression of chIFITM1 in LMH cells promotes the replication of IBV Beaudette strains, which was surprising because IFITM1 in most cases exhibits inhibitory effects on viruses, especially enveloped viruses. Next, we continued to use other viruses (NDV and FADV) to infect LMH cells overexpressing chIFITM1. The results showed that the overexpression of IFITM1 exerted no effect on NDV replication, but slightly promoted FADV infection in mRNA level (Fig. 2D). These results indicated that the function of IFITM1 is intricately related to virus and cell type.

chIFITM1 and huIFITM1 are homologous proteins and share similar transmembrane structures. The IBV Beaudette strain has been demonstrated to replicate in a variety of human cell lines, such as Huh7, H1299, HepG2, and Hep3B. To explore the interaction between huIFITM1 and IBV Beaudette infection in a human cell line, huIFITM1 was overexpressed in Huh7 cells, followed by infection with IBV Beaudette. The results showed that chIFITM1 huIFITM were correctly expressed in Huh7 cells (SUPPLEMENTARY MATERIAL 1), and huIFITM1 significantly inhibited the infection of the IBV Beaudette strain in Huh7 cells. Interestingly, the overexpression of chIFITM1 (sequence not optimized) effectively inhibited IBV replication. The degree of inhibition was even higher than that of huIFITM1 (Fig. 2E-F). These results further indicated that the function of IFITM1 is intricately related to cell type.

3.3 chIFITM1 does not affect viral attachment and entry

It has been reported that IFITM1 restricts viral infection primarily by affecting the attachment of viral particles to cellular receptors or by affecting the fusion of viral membranes with cellular membranes [27]. As an enveloped virus, IBV primarily utilizes clathrin-mediated endocytosis (CME) for entry, viral particles move along the classical endosome/lysosome track and release the viral genome at late endosome/lysosome stage [28]. To assess whether chIFITM1 functions in the early stages of IBV infection, viral particles binding and entering overexpressed chIFITM1 LMH cells. No significant difference was observed at the IBV mRNA level between the treatment and negative control groups (Fig. 3A, B). It indicated that the overexpression of IFITM1 in chicken LMH cells did not interfere

with IBV attachment and entry. Previous results showed that the viral mRNA in cells overexpressing IFITM1 was considerably higher than that in the control group, suggesting that IFITM1 may promote IBV replication in the cytoplasm.

3.4 Subcellular localization of chIFITM1/2/3 on chicken cells

Correct positioning forms the basis for proteins to function. In mammalian cells, IFITM1 is primarily localized at the plasma membrane, whereas IFITM2 and IFITM3 are found inside the cell on endo-lysosomal membranes. To confirm if different functions of chIFITMs in different cells are attributed to their subcellular localization, HD11 and LMH cells were transfected with chIFTM1/2/3 and the endosomal markers chRab5a, respectively. DF-1 cells were not discussed in subsequent experiments due to the limited effect of IFITMs in IBV-infected DF-1 cells. Confocal analysis results showed that in HD11 cells, the majority of chIFITM1/2 was located on the cell membrane and little in the cytoplasm, whereas, chIFITM3 was largely co-localized with chRab5a in the cytoplasm (Fig. 4A). Similar to HD11 cells, chIFITMs share the same location in LMH cells as in HD11 cells (Fig. 4B). These results indicated that the subcellular localization of chIFITMs was approximately the same in the two cells, suggesting that different functions of chIFITM1 were unlikely to be caused by its localization.

3.5 Intracellular loop domain (CIL) is essential for chIFITM1 to promote viral proliferation

IFITMs are generally encoded by two exons [29]. All IFITMs belong to a class of twice-transmembrane proteins, with an N-terminal domain (NTD), two transmembrane domains (TMD), and an intracellular loop domain (CIL), and an C-terminal domain (CTD) domains[3]. Among them, the component consisting of two TMDs and one CIL is defined as the CD225 domain, which is relatively conserved among different species. Intracellular domains can interact directly with intracellular proteins to transmit the resulting signals deeper into the cells. It is still unknown whether such a pattern is required in chIFITM1 in promoting viral infection

We predicted the transmembrane structure of chIFITM1 using TMHMM-2.0 and found that it followed the structural features of mammalian IFITMs (Fig 5A). chIFITM1 was mutated with four consecutive alanine residues to identify key locations through which chIFITM1 promotes viral replication (Fig. 5B). The results showed that mutations in two regions, DN60-63 (KSRD) and DN68-71 (KDFV), resulted in a loss of "promoting function", whereas other mutations exerted no significant effect on IBV replication (Fig. 5C). Furthermore, we found that mutations in both regions, DN60-63 (KSRD) and DN68-71 (KDFV), did not alter the subcellular localization of chIFITM1. The mutant chIFITM1 still functions as a membrane protein (Fig. 5D). Therefore, we hypothesized there were other proteins, interacted with IFITM1 (especially the CLD), involved in the process of co-promoting IBV proliferation.

3.6 Pathway analysis of chIFITM1 interaction protein

All intracellular proteins are implicated in complex and precise regulatory networks. However, studies on the regulatory network of IFITMs are rare. Here, we used chIFITM1 as bait to pull down interacting proteins. The results showed that more than 100 proteins were identified in HD11 cells, and more than 250 proteins were identified in LMH cells (SUPPLEMENTARY MATERIAL 2). For example, aREEP5, SEC61B, EIF4B, ATP5H, RAB8A, and EIF3H were detected in both cell types. However, several proteins, such as HSP90AB1, CTNNB1, ATP5F1AZ, and HSPB9, were only detected in LMH cells. GO categorization and STRING were used in target protein enrichment analysis. The top 10 GO significantly enriched terms are shown in Fig. 6A-B. This revealed that the identified proteins were largely involved in single-organism cellular process, single-organism location, and single-organism location transport in HD11. However, identified proteins in LMH were largely involved in *single-organism process, organic substance metabolic process and single-organism cellular process*. Cell components showed that the identified proteins were predominantly intracellular in both cells. The results of molecular function demonstrated that the obtained proteins were largely involved in nucleic acid attachment. Several proteins in LMH were enriched in *organic cyclic compound attachment* terms. Overall, the proteins obtained in the two cell types were partly the same; however, most of them were different, indicating that IFITM1 could be involved in different regulatory pathways in different cells. We constructed the chIFITM1 protein interaction landscape in HD11 and LMH cells using STRING (Fig. 6C-D). The results showed that these proteins formed a complex interaction network in both types of cells. For example, ABCE1 can interact with up to 38 proteins in LMH cells. Similarly, EPRS can interact with nine proteins in HD11 cells. A complex regulatory network could lead to different roles of chIFITM1 for viral infection in the two types of cells. Such a conclusion should be drawn with caution because most STRING predictions are based on mammals, and certain chicken genes have not been particularly well annotated. Therefore, the actual interaction relationship needs to be further verified by experiments such as co-immunoprecipitation (co-IP).

4. Discussion

It is reported that IFITM proteins restrict infection by several viruses, including enveloped viruses and non-enveloped viruses, DNA viruses, and RNA viruses. However, in this study, we revealed that IFITMs have different functions in different cells. In particular, chIFITM1 promoted the replication of certain viruses in LMH cells, which was diametrically opposed to the conclusions of several reports. We showed that the subcellular localization of chIFITMs in different cells is roughly the same. chIFITM1/2 are largely located in the cell membrane, with chIFITM3 located in the endosomal membrane. The overexpression of chIFITM1 did not affect IBV attachment and entry. Furthermore, the intracellular loop is essential for the function of chIFITM1. The interaction with different downstream proteins in different cells could be the primary reason why IFITM plays different roles.

IBV is a chicken coronavirus that is known to seriously threaten the poultry industry. All wild-type IBV strains were unable to passage through cell lines. IBV Beaudette is the only cell-adapted strain that can be passaged in two avian cell lines, i.e., DF-1 and HD11 and several mammalian cell lines [30]. Here, we showed that the Beaudette strain can be stably replicated in the LMH cell line. LMH was the first established domestic fowl epithelial cell line [31] in which IBV replicates extremely vigorously such that the titer exceeds that obtained in Vero cells (data not shown), thereby providing suitable material for future research. We overexpressed chIFITM1 in several chicken cells and observed the effect of this treatment on IBV. The results indicated that the responses of chIFITM1 to IBV infection in HD11 and LMH cells were diametrically opposite. It is not reported for the first time that IFITMs have different responses to viruses in different cell types. A recent study showed that the overexpression of IFITM2 in SLK cells slightly enhanced Kaposi's Sarcoma-Associated Herpesvirus (KSHV) infection and significantly enhanced Related Rhesus Monkey Rhadinovirus (RRV) infection [32]. Similarly, the overexpression of IFITM1 and IFITM3 consistently enhanced HPV infectivity in several epithelial cell lines and keratinocytes [21]. Furthermore, IFITM1 expression enhanced the KSHV, EBV, and HSV-2 infection of BJAB and HMVEC-d cells [33].

Although the detailed molecular mechanism underlying the ability of IFITMs to restrict viral infection is still unclear, most studies report that IFITM exerts its effect by affecting the cell membrane. This is largely attributed to the localization of IFITMs on cell membranes or endosomal membranes [34]. We compared the subcellular localization of chIFITM1/2/3 in HD11 and LMH cells and found that chIFITM1/2 was mostly located in the cell membrane, whereas chIFITM3 was located in the cytoplasm and co-localized with Rab5a. The subcellular localization of IFITMs in both cells was almost identical. The results for chIFITM2/3 were consistent with those of previous studies; however, the results for chIFITM1 were different. Two research groups [4; 35] reported that chIFITM1 is diffusely expressed throughout the cytoplasm; however, our results showed that most of it were distributed on the membrane. This could be ascribed to different cell lines or different tags (Its effect on small proteins should not be ignored). In addition, due to the lack of antibodies, this study used exogenous overexpression of chIFITMs; high levels of exogenously expressed protein could have resulted in abnormal localization and function of the protein to a certain extent [32; 36]. Studies have demonstrated that endogenous IFITM can promote SARS-CoV-2 virus infection, whereas exogenously expressed IFITM exerts the opposite effect [37; 38]. Therefore, these *in vitro* experiments provide limited evidence for *in vivo* studies.

Previous studies have demonstrated that the CIL region of IFITM1 is necessary for restriction [27; 29]. Here, alanine scan mutants of the CIL were constructed to explore the

contribution of the key region to the IFITM function. We showed that two domains, namely, "KDFV" and "KSRD" are necessary for the "promoting" effect of chIFITM1 on IBV, which is consistent with the finding of a previous study [29]. Sun's study [39] demonstrated that the "KRRK" motif is necessary for limiting viral infection, whereas our study demonstrated that this region in chIFITM1 is necessary for "promoting" viral infection, suggesting the significance of this region for the basal function of IFITM. As a transmembrane protein, the intracellular domain of IFITM could be responsible for transmitting signals within cells. Several upstream studies on the activation or regulation of IFITM are available [40]. However, records of IFITM-interacting proteins are sparse. Maria et al. [41] studied the interaction of proteins with IFITM1 using SWATH-IP mass spectrometry. We also assayed the proteins interacting with chIFITM1 in both cell types and showed that these proteins can form complex networks. The networks in HD11 and LMH cells were highly different, reflecting that chIFITM1 could interact with different downstream proteins in different cells. Especially, with regard to LMH as a cancer cell, an increasing number of studies have demonstrated the important function of IFITM1 in carcinogenesis [42; 43; 44]. IFITM1 promotes tumor cell proliferation, inhibits cell death, stimulates invasion and metastasis, and has been regarded as an independent prognostic biomarker for patients with certain tumor types, such as gallbladder carcinoma, esophageal adenocarcinoma, colorectal cancer, and gastric cancer [45]. The regulatory network of IFITM1 in cancer cells is highly complex, and the relationship between IFITM1 and viral infection in cancer cells requires additional attention.

In addition, the function of IFITMs is intricately related to the pathway through which the virus enters the cell. As an enveloped coronavirus, IBV entry is primarily dependent on clathrin-mediated endocytosis and requires the classical endosomal/lysosomal system [28]. However, virus entry into different cell lines may involve different pathways, the pathways used by IBV to enter LMH and HD11 cells are still unclear. Moreover, correct modifications, such as palmitoylation, ubiquitination, phosphorylation and methylation, are required for IFITM to function [46; 47; 48; 49]. It remains to be verified whether the modifications of chIFITM1 in different cells are the primary reason for the differences.

In conclusion, this study reports the different functions of chIFITMs in different chicken cell lines. The subcellular localization of chIFITM1/2/3 in HD11 and LMH cells was largely the same. chIFITM1/2 was largely located in the cell membrane, whereas chIFITM3 was located in the cytoplasm. The correct amino acid sequence of CIL region is necessary for the basal function of chIFITM1. In addition, the network of proteins interacting with chIFITM1 in different cells, especially cancer cells, is extremely different and complex and is the primary reason for *in vitro* studies affecting the function of IFITM1. The above results provide new insights into our understanding of the role of IFITMs in coronavirus infection.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: HL, RQL and XY. **Methodology:** HL, YMT and RQL. **Validation:** HL and RQL. **Formal Analysis:** RQL, HLG and KLW. **Investigation:** RQL, HLG and KLW. **Writing–Original Draft:** HL and RQL. **Review & Editing:** XY, CWL and YZT. **Visualization:** HL and WJY. **Supervision:** XY, HNW, CWL and YZT. **Project Administration:** XY and HNW. **Funding Acquisition:** XY and HNW. HL and RQN contributed equally to the article. All authors contributed to the article and approved the submitted version.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Reference

[1] R.L. Friedman, S.P. Manly, M. Mcmahon, I.M. Kerr, and G.R. Stark, Transcriptional and Posttranscriptional Regulation of Interferon-Induced Gene-Expression in Human-Cells. Cell 38 (1984) 745-755.

[2] S.S. Tanaka, Y.L. Yamaguchi, B. Tsoi, H. Lickert, and P.P.L. Tam, IFITM/mil/fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion. Developmental Cell 9 (2005) 745-756.

[3] Y. Liao, M.U. Goraya, X. Yuan, B.G. Zhang, S.H. Chiu, and J.L. Chen, Functional Involvement of Interferon-Inducible Transmembrane Proteins in Antiviral Immunity. Frontiers in Microbiology 10 (2019).

[4] S.E. Smith, M.S. Gibson, R.S. Wash, F. Ferrara, E. Wright, N. Temperton, P. Kellam, and M. Fife, Chicken Interferon-Inducible Transmembrane Protein 3 Restricts Influenza Viruses and Lyssaviruses In Vitro. J Virol 87 (2013) 12957-12966.

[5] Y. Okuzaki, S. Kidani, H. Kaneoka, S. Iijima, and K. Nishijima, Characterization of chicken interferon-inducible transmembrane protein-10. Biosci Biotech Bioch 81 (2017) 914-921.

[6] H. Hatano, Y. Kudo, I. Ogawa, T. Tsunematsu, A. Kikuchi, Y. Abiko, and T. Takata, IFN-Induced Transmembrane Protein 1 Promotes Invasion at Early Stage of Head and Neck Cancer Progression. Clin Cancer Res 14 (2008) 6097-6105.

[7] J. Yang, L. Li, Y. Xi, R.M. Sun, H. Wang, Y.X. Ren, L.F. Zhao, X.L. Wang, and X.J. Li, Combination of IFITM1 knockdown and radiotherapy inhibits the growth of oral cancer. Cancer Sci 109 (2018) 3115-3128.

[8] J.Z. Li, N. Chen, and X.B. Gong, Prognostic implications of aberrantly expressed methylation-driven genes in hepatocellular carcinoma: A study based on The Cancer Genome Atlas. Mol Med Rep 20 (2019) 5304-5314.

[9] G.A.D. Blyth, W.F. Chan, R.G. Webster, and K.E. Magor, Duck Interferon-Inducible Transmembrane Protein 3 Mediates Restriction of Influenza Viruses. J Virol 90 (2016) 103-116.

[10] A.L. Brass, I.C. Huang, Y. Benita, S.P. John, M.N. Krishnan, E.M. Feeley, B.J. Ryan, J.L. Weyer, L. van der Weyden, E. Fikrig, D.J. Adams, R.J. Xavier, M. Farzan, and S.J. Elledge, The IFITM Proteins Mediate Cellular Resistance to Influenza A H1N1 Virus, West Nile Virus, and Dengue Virus. Cell 139 (2009) 1243-1254.

[11] D. Jiang, J.M. Weidner, M. Qing, X.B. Pan, H.T. Guo, C.X. Xu, X.C. Zhang, A. Birk, J.H. Chang, P.Y. Shi, T.M. Block, and J.T. Guo, Identification of Five Interferon-Induced Cellular Proteins That Inhibit West Nile Virus and Dengue Virus Infections. J Virol 84 (2010) 8332-8341.

[12] I.C. Huang, C.C. Bailey, J.L. Weyer, S.R. Radoshitzky, M.M. Becker, J.J. Chiang, A.L. Brass, A.A. Ahmed, X.L. Chi, L.A. Dong, L.E. Longobardi, D. Boltz, J.H. Kuhn, S.J. Elledge, S. Bavari, M.R. Denison, H. Choe, and M. Farzan, Distinct Patterns of IFITM-Mediated Restriction of Filoviruses, SARS Coronavirus, and Influenza A Virus. Plos Pathog 7 (2011).

[13] G.L. Shi, A.D. Kenney, E. Kudryashova, A. Zani, L.Z. Zhang, K.K. Lai, L. Hall-Stoodley, R.T. Robinson, D.S. Kudryashov, A.A. Compton, and J.S. Yount, Opposing activities of IFITM proteins in SARS-CoV-2 infection. Embo J 40 (2021).

[14] S.L. Chen, L. Wang, J.Y. Chen, L.L. Zhang, S. Wang, M.U. Goraya, X.J. Chi, Y. Na, W.H. Shao, Z. Yang, X.C. Zeng, S.Y. Chen, and J.L. Chen, Avian Interferon-Inducible Transmembrane Protein Family Effectively Restricts Avian Tembusu Virus Infection. Front Microbiol 8 (2017).

[15] M.A. Rohaim, M.Q. Al-Natour, M.A. Abdelsabour, R.F. El Naggar, Y.M. Madbouly, K.A. Ahmed, and M. Munir, Transgenic Chicks Expressing Interferon-Inducible Transmembrane Protein 1 (IFITM1) Restrict Highly Pathogenic H5N1 Influenza Viruses. Int J Mol Sci 22 (2021).

[16] A. Steyn, S. Keep, E. Bickerton, and M. Fife, The Characterization ofchIFITMsin Avian Coronavirus Infection In Vivo, Ex Vivo and In Vitro. Genes-Basel 11 (2020).

[17] X.S. Zhao, J.R. Li, C.A. Winkler, P. An, and J.T. Guo, IFITM Genes, Variants, and Their Roles in the Control and Pathogenesis of Viral Infections. Front Microbiol 9 (2019).

[18] A.A. Anafu, C.H. Bowen, C.R. Chin, A.L. Brass, and G.H. Holm, Interferon-inducible Transmembrane Protein 3 (IFITM3) Restricts Reovirus Cell Entry. J Biol Chem 288 (2013) 17261-17271.

[19] J.F. Xu, P. Qian, Q.F. Wu, S.S. Liu, W.C. Fan, K.S. Zhang, R. Wang, H.W. Zhang, H.C. Chen, and X.M. Li, Swine interferon-induced transmembrane protein, sIFITM3, inhibits foot-and-mouth disease virus infection in vitro and in vivo. Antivir Res 109 (2014) 22-29.

[20] X.S. Zhao, F. Guo, F. Liu, A. Cuconati, J.H. Chang, T.M. Block, and J.T. Guo, Interferon induction of IFITM proteins promotes infection by human coronavirus OC43. P Natl Acad Sci USA 111 (2014) 6756-6761.

[21] C.J. Warren, L.M. Griffin, A.S. Little, I.C. Huang, M. Farzan, and D. Pyeon, The Antiviral Restriction Factors IFITM1, 2 and 3 Do Not Inhibit Infection of Human Papillomavirus, Cytomegalovirus and Adenovirus. Plos One 9 (2014).

[22] C.P. Bozzo, R. Nchioua, M. Volcic, L. Koepke, J. Kruger, D. Schutz, S. Heller, C.M. Sturzel, D. Kmiec, C. Conzelmann, J. Muller, F. Zech, E. Braun, R. Gross, L. Wettstein, T. Weil, J. Weiss, F. Diofano, A.A.R. Alfonso, S. Wiese, D. Sauter, J. Munch, C. Goffinet, A. Catanese, M. Schon, T.M. Boeckers, S. Stenger, K. Sato, S. Just, A. Kleger, K.M.J. Sparrer, and F. Kirchhoff, IFITM proteins promote SARS-CoV-2 infection and are targets for virus inhibition in vitro. Nat Commun 12 (2021).

[23] X.W. Zhai, X.R. Mei, X. Wu, L. Zuo, L. Zhou, Y.M. Tian, X.X. Han, X. Yang, and H.N. Wang, A loop-mediated isothermal amplification coupling with a lateral flow dipstick for rapid and specific detection of fowl adenovirus serotype-4. J Virol Methods 270 (2019) 79-86.

[24] X. Wu, X.W. Zhai, Y. Lai, L. Zuo, Y. Zhang, X.R. Mei, R. Xiang, Z.Z. Kang, L.Z. Zhou, and H.N. Wang, Construction and Immunogenicity of Novel Chimeric Virus-Like Particles Bearing Antigens of Infectious Bronchitis Virus and Newcastle Disease Virus. Viruses-Basel 11 (2019).

[25] R. Guan, Y.M. Tian, X.X. Han, X. Yang, and H.N. Wang, Complete genome sequence and pathogenicity of fowl adenovirus serotype 4 involved in hydropericardium syndrome in Southwest China. Microb Pathogenesis 117 (2018) 290-298.

[26] X.X. Han, Y.M. Tian, R. Guan, W.Q. Gao, X. Yang, L. Zhou, and H.N. Wang, Infectious Bronchitis Virus Infection Induces Apoptosis during Replication in Chicken Macrophage HD11 Cells. Viruses-Basel 9 (2017).

[27] S.E. Smith, D.C. Busse, S. Binter, S. Weston, C.D. Soria, B.M. Laksono, S. Clare, S. Van Nieuwkoop, B.G. Van den Hoogen, M. Clement, M. Marsden, I.R. Humphreys, M. Marsh, R.L. de Swart, R.S. Wash, J.S. Tregoning, and P. Kellam, Interferon-Induced Transmembrane Protein 1 Restricts Replication of Viruses That Enter Cells via the Plasma Membrane. J Virol 93 (2019).

[28] H. Wang, X. Yuan, Y.J. Sun, X. Mao, C.C. Meng, L. Tan, C.P. Song, X.S. Qiu, C. Ding, and Y. Liao, Infectious bronchitis virus entry mainly depends on clathrin mediated endocytosis and requires classical endosomal/lysosomal system. Virology 528 (2019) 118-136.

[29] F. Sun, Z.Q. Xia, Y.W. Han, M.J. Gao, L.Y. Wang, Y.L. Wu, J.M. Sabatier, L.X. Miao, and Z.J. Cao, Topology, Antiviral Functional Residues and Mechanism of IFITM1. Viruses-Basel 12 (2020).

[30] F.P.L. Tay, M. Huang, L. Wang, Y. Yamada, and D.X. Liu, Characterization of cellular furin content as a potential factor determining the susceptibility of cultured human and animal cells to coronavirus infectious bronchitis virus infection. Virology 433 (2012) 421-430.

[31] T. Kawaguchi, K. Nomura, Y. Hirayama, and T. Kitagawa, Establishment and Characterization of a Chicken Hepatocellular-Carcinoma Cell-Line, Lmh. Cancer Res 47 (1987) 4460-4464.

[32] B.F. Hornich, A.K. Grosskopf, C.J. Dcosta, S. Schlagowski, and A.S. Hahn, Interferon-Induced Transmembrane Proteins Inhibit Infection by the Kaposi's Sarcoma-Associated Herpesvirus and the Related Rhesus Monkey Rhadinovirus in a Cell-Specific Manner. mBio 12 (2021) e0211321.

[33] H.A.M. Hussein, and S.M. Akula, miRNA-36 inhibits KSHV, EBV, HSV-2 infection of cells via stifling expression of interferon induced transmembrane protein 1 (IFITM1). Sci Rep 7 (2017) 17972.

[34] M.S. Diamond, and M. Farzan, The broad-spectrum antiviral functions of IFIT and IFITM proteins. Nat Rev Immunol 13 (2013) 46-57.

[35] R. Zhao, Q.K. Shi, Z.X. Han, Z. Fan, H. Ai, L.N. Chen, L. Li, T.Y. Liu, J.F. Sun, and S.W. Liu, Newcastle Disease Virus Entry into Chicken Macrophages via a pH-Dependent, Dynamin and Caveola-Mediated Endocytic Pathway That Requires Rab5. J Virol 95 (2021).

[36] C.C. Bailey, G. Zhong, I.C. Huang, and M. Farzan, IFITM-Family Proteins: The Cell's First Line of Antiviral Defense. Annu Rev Virol 1 (2014) 261-283.

[37] C. Prelli Bozzo, R. Nchioua, M. Volcic, L. Koepke, J. Kruger, D. Schutz, S. Heller, C.M. Sturzel, D. Kmiec, C. Conzelmann, J. Muller, F. Zech, E. Braun, R. Gross, L. Wettstein, T. Weil, J. Weiss, F. Diofano, A.A. Rodriguez Alfonso, S. Wiese, D. Sauter, J. Munch, C. Goffinet, A. Catanese, M. Schon, T.M. Boeckers, S. Stenger, K. Sato, S. Just, A. Kleger, K.M.J. Sparrer, and F. Kirchhoff, IFITM proteins promote SARS-CoV-2 infection and are targets for virus inhibition in vitro. Nat Commun 12 (2021) 4584.

[38] G. Shi, A.D. Kenney, E. Kudryashova, A. Zani, L. Zhang, K.K. Lai, L. Hall-Stoodley, R.T. Robinson, D.S. Kudryashov, A.A. Compton, and J.S. Yount, Opposing activities of IFITM proteins in SARS-CoV-2 infection. EMBO J 40 (2021) e106501.

[39] F. Sun, Z. Xia, Y. Han, M. Gao, L. Wang, Y. Wu, J.M. Sabatier, L. Miao, and Z. Cao, Topology, Antiviral Functional Residues and Mechanism of IFITM1. Viruses 12 (2020).

[40] S. Wang, X.J. Chi, H.T. Wei, Y.H. Chen, Z.L. Chen, S.L. Huang, and J.L. Chen, Influenza A Virus-Induced Degradation of Eukaryotic Translation Initiation Factor 4B Contributes to Viral Replication by Suppressing IFITM3 Protein Expression. J Virol 88 (2014) 8375-8385.

[41] M. Gomez-Herranz, M. Nekulova, J. Faktor, L. Hernychova, S. Kote, E.H. Sinclair, R. Nenutil, B. Vojtesek, K.L. Ball, and T.R. Hupp, The effects of IFITM1 and IFITM3 gene deletion on IFN gamma stimulated protein synthesis. Cell Signal 60 (2019) 39-56.

[42] A. Kelemen, I. Carmi, A. Oszvald, P. Lorincz, G. Petovari, T. Tolgyes, K. Dede, A. Bursics, E.I. Buzas, and Z. Wiener, IFITM1 expression determines extracellular vesicle uptake in colorectal cancer. Cell Mol Life Sci 78 (2021) 7009-7024.

[43] O.K. Provance, E.S. Geanes, A.J. Lui, A. Roy, S.M. Holloran, S. Gunewardena, C.R. Hagan, S. Weir, and J. Lewis-Wambi, Disrupting interferon-alpha and NF-kappaB crosstalk suppresses IFITM1 expression attenuating triple-negative breast cancer progression. Cancer Lett 514 (2021) 12-29.

[44] S. Sakamoto, H. Inoue, Y. Kohda, S. Ohba, T. Mizutani, and M. Kawada, Interferon-Induced Transmembrane Protein 1 (IFITM1) Promotes Distant Metastasis of Small Cell Lung Cancer. Int J Mol Sci 21 (2020).

[45] R.B. Liang, X.X. Li, and X.D. Zhu, Deciphering the Roles of IFITM1 in Tumors. Mol Diagn Ther 24 (2020) 433-441.

[46] Z. Xu, X.L. Li, J.C. Xue, L.Y. Shen, W.M. Zheng, S.G. Yin, and J. Xu, S-palmitoylation of swine interferon-inducible transmembrane protein is essential for its anti-JEV activity. Virology 548 (2020) 82-92.

[47] J.S. Yount, B. Moltedo, Y.Y. Yang, G. Charron, T.M. Moran, C.B. Lopez, and H.C. Hang, Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. Nat Chem Biol 6 (2010) 610-614.

[48] N.M. Chesarino, T.M. McMichael, J.C. Hach, and J.S. Yount, Phosphorylation of the Antiviral Protein Interferon-inducible Transmembrane Protein 3 (IFITM3) Dually Regulates Its Endocytosis and Ubiquitination. J Biol Chem 289 (2014) 11986-11992.

[49] Z. Shan, Q.L. Han, J. Nie, X.Z. Cao, Z.J. Chen, S.Y. Yin, Y.Y. Gao, F. Lin, X.H. Zhou, K. Xu, H.M. Fan, Z.K. Qian, B. Sun, J. Zhong, B. Li, and A. Tsun, Negative Regulation of Interferon-induced Transmembrane Protein 3 by SET7-mediated Lysine Monomethylation. J Biol Chem 288 (2013) 35093-35103.

[50] S. Moller, M.D.R. Croning, and R. Apweiler, Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17 (2001) 646-653.

Figure 1. Susceptibility of LMH cells to Beaudette strain. (A) IBV Beaudette strain serial propagation for 20 passages on LMH cells. Distinct bands were detected at every passage after electrophoresis using agarose gel electrophoresis. Primers were designed in the conserved 3'-UTR region (F: TTGCTGGTATCACTGCTTGTT R: GCCATGTTGTCACTGTCTATTG). (B) Cell pathological effects (CPE) of IBV-infected LMH cells. Red arrows identify syncytia (amplification: 100×). (C) An indirect immunofluorescence assay (IFA) was performed to detect IBV infection status in LMH cells. Cells were fixed at the indicated time, and virally infected cells were visualized by immunofluorescence (IF) staining of the IBV N protein (green). Cell nuclei were visualized by DAPI staining (blue). Merge is an overlap of DAPI and NP (amplification: 200×).

Figure 2. Effect of overexpression of IFITMs in diferent cells on IBV or other avian virus replication. Virus-infected or noninfected cell lysates were collected at 24h post-transfection. The relative expression of the IBV N gene was determined by RT-qPCR or western blotting, using chGAPDH or huGAPDH as the reference (*p < 0.5; **p < 0.01; ****p < 0.0001). Data represent the mean and SD of three independent experiments. (A) The expression level of IBV mRNA and N protein in chIFITMs-overexpressed HD11 cells. (B) The expression level of IBV mRNA and N protein in chIFITMs-overexpressed DF-1 cells. (C) The expression level of IBV mRNA and N protein in chIFITMs-overexpressed LMH cells. (D) The expression levels of nucleic acids of different kinds of viruses (IBV, NDVand FADV) in overexpressed-chIFITM1 LMH cells, respectively. (E-F) The expression level of IBV mRNA or IBV N protein in overexpressed chIFITM1 or huIFITM1 Huh7 cells, using huGAPDH as the reference.

Figure 3. Effect of overexpression chIFITM1/2/3 on IBV attachment and entry. Cells were transfected with chIFITM1/2/3 and inoculated with IBV (MOI = 10) at 4°C for 1 h and subsequently shifted to 37°C. At 0 (attachment) or 1 (entry) hpi, infected cells were lysed to determine the virus mRNA levels by RT-qPCR. (A) The mRNA expression levels of IBV attached on the surface of chIFITMs-overexpressed LMH cells. (B) The mRNA expression levels of IBV entred into the chIFITMs-overexpressed LMH cells.

Figure 4. Cellular localization of chIFITM1/2/3 proteins in chicken cell lines. Cells were transfected with chIFITM1/2/3 and chRab5a 24 h before fixation. The position of chRab5a and chIFITM1/2/3 are shown by GFP (green) and flag (red) staining, respectively. Cell nuclei were visualized by DAPI staining (blue). Merge is an overlap of GFP, flag, and DAPI. (A) Subcellular localization of chIFITM1/2/3 in HD11 cells. (B) Subcellular localization of chIFITM1/2/3 in LMH cells.

Figure 5. Key site for chIFITM1 to promote viral replication. (A) Structural model of chIFITM1 on the cell membrane, predicted using TMHMM-2.0 [50]. (B) Schematic diagram of alanine scanning mutagenesis used in this study. WT is wild-type chIFITM1, and the numbers represent the positions of mutated amino acids. (C) Virus-infected cell lysates were collected at 24h post-transfection. The IBV N expression levels in chIFITM1 or various mutant chIFITM1-overexpressed LMH cells were determined by western blotting, using chGAPDH as the reference. (D) Subcellular localization of DN-chIFITM1 (60KSRD63, 68KDFV71) in LMH cells.

Figure 6. Bioinformatics analysis of proteins interacting with chIFITM1 in HD11 and LMH. (A, B) Gene ontology (GO) analysis of proteins interacting with chIFITM1 in HD11 and LMH. (C, D) STRING (protein–protein interaction analysis) of proteins interacting with chIFITM1 in HD11 and LMH. The node represents the protein name, and the line represents the interaction relationship.

Figure 1

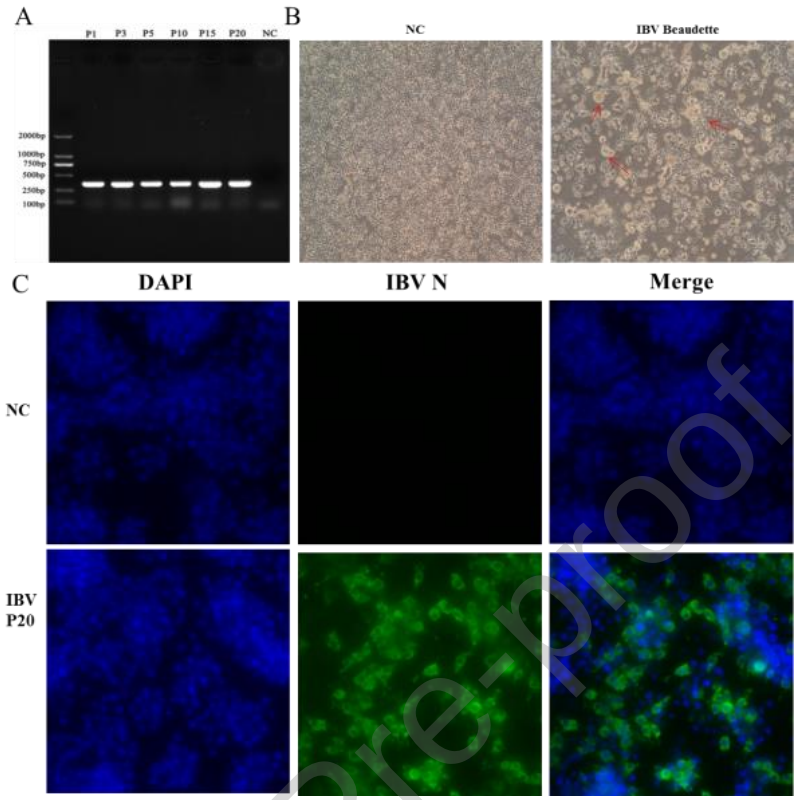


Figure 2

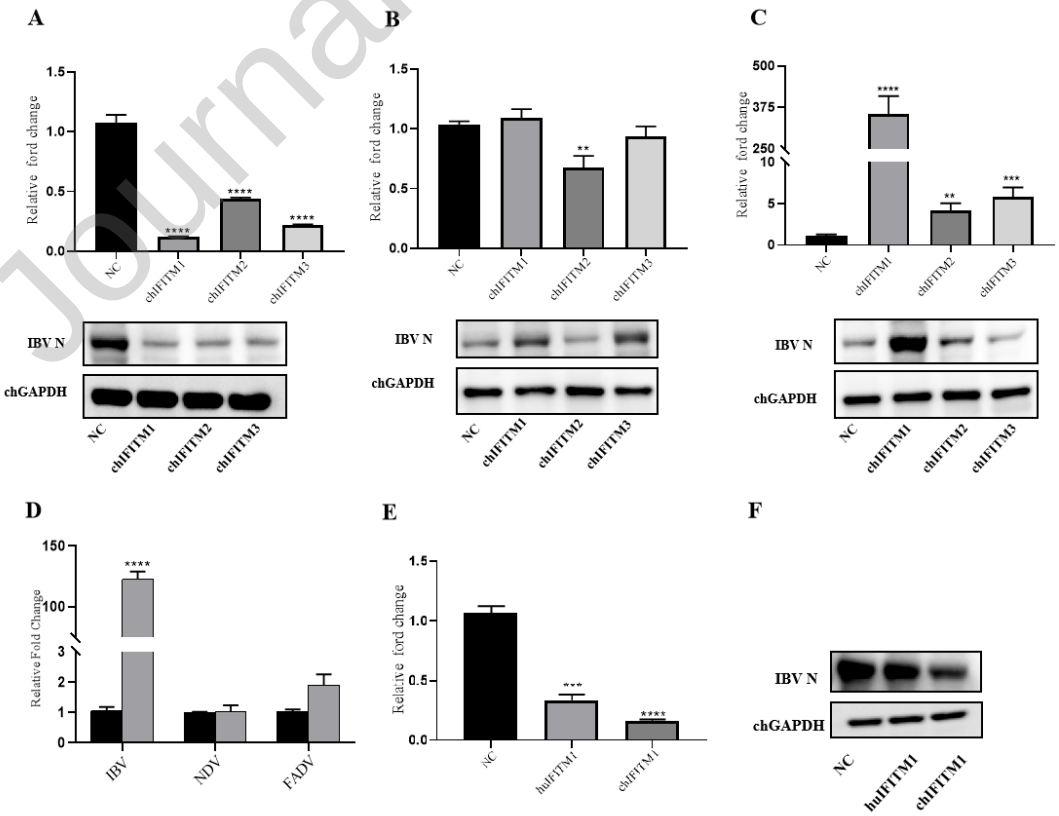


Figure 3

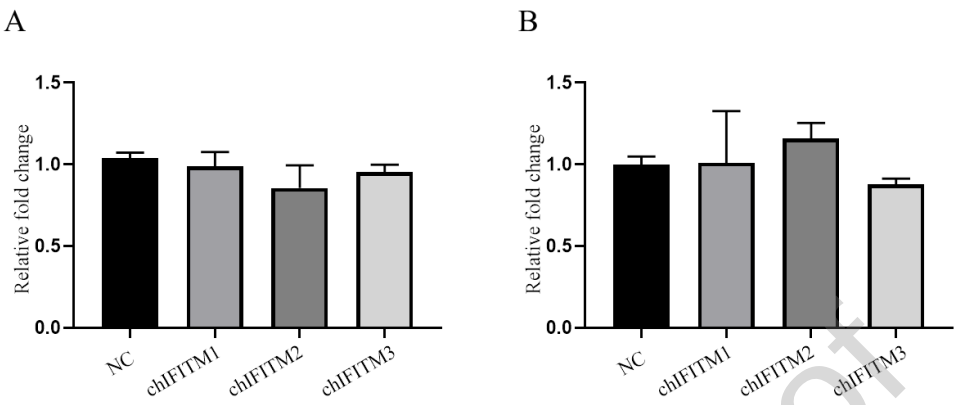
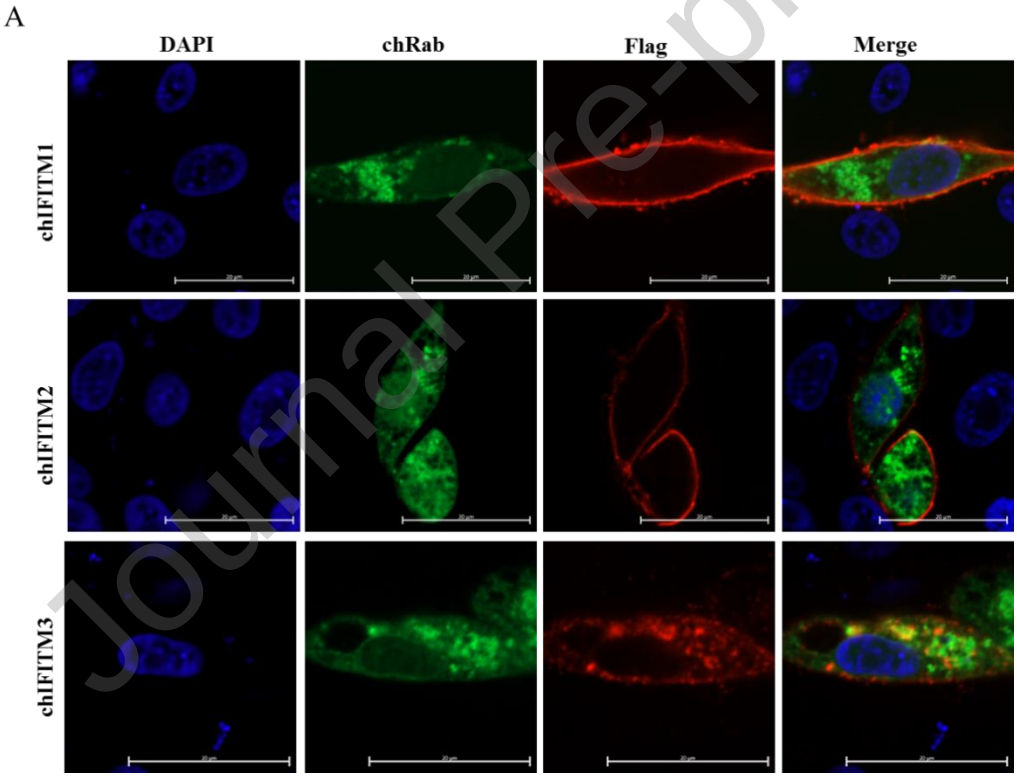


Figure 4



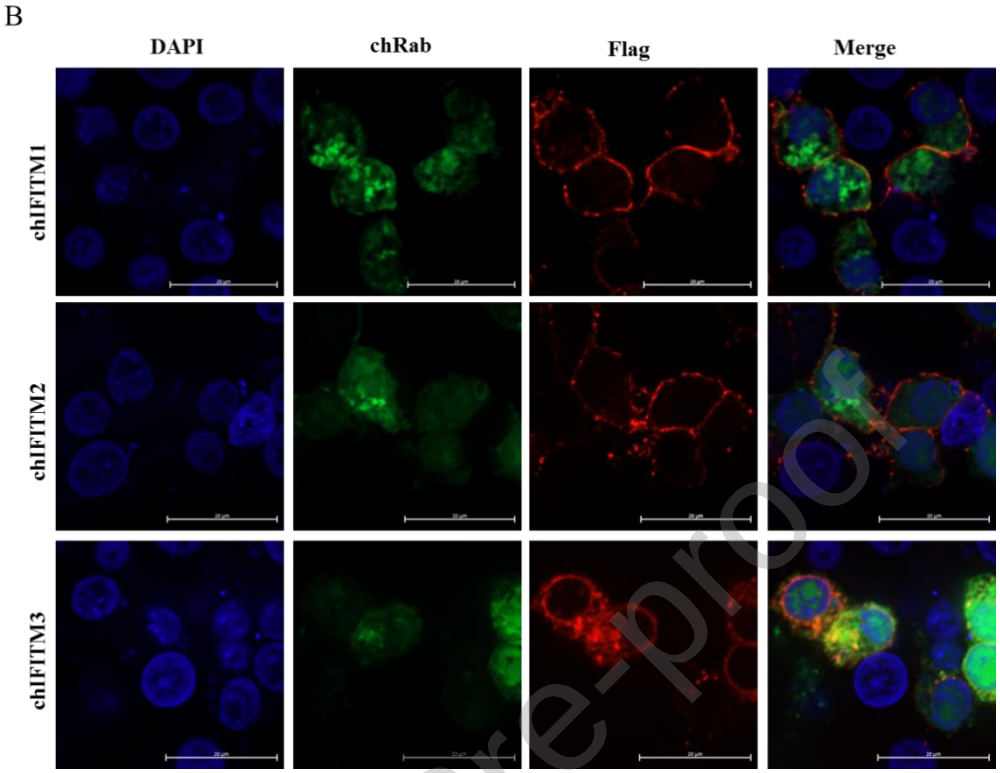


Figure 5

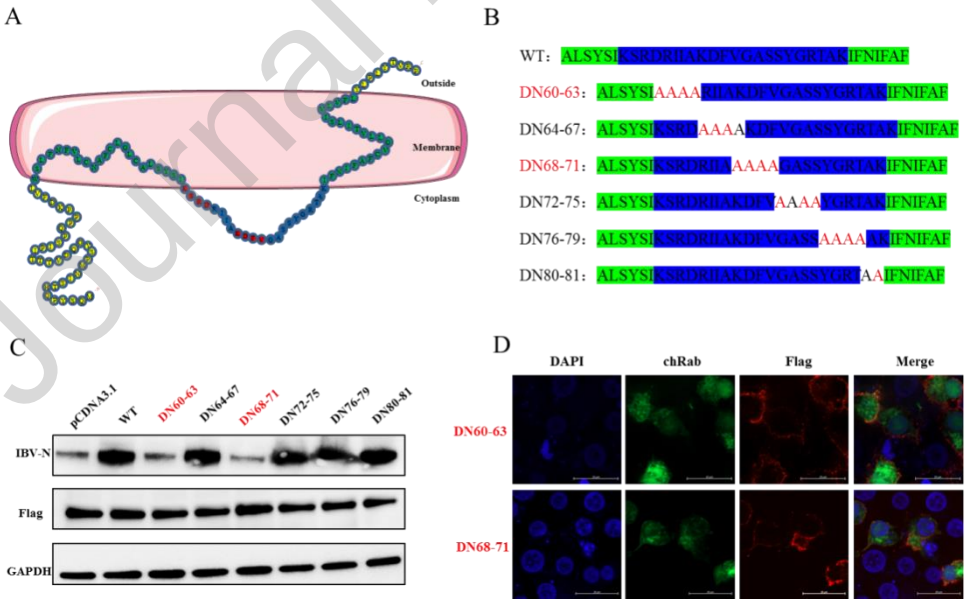
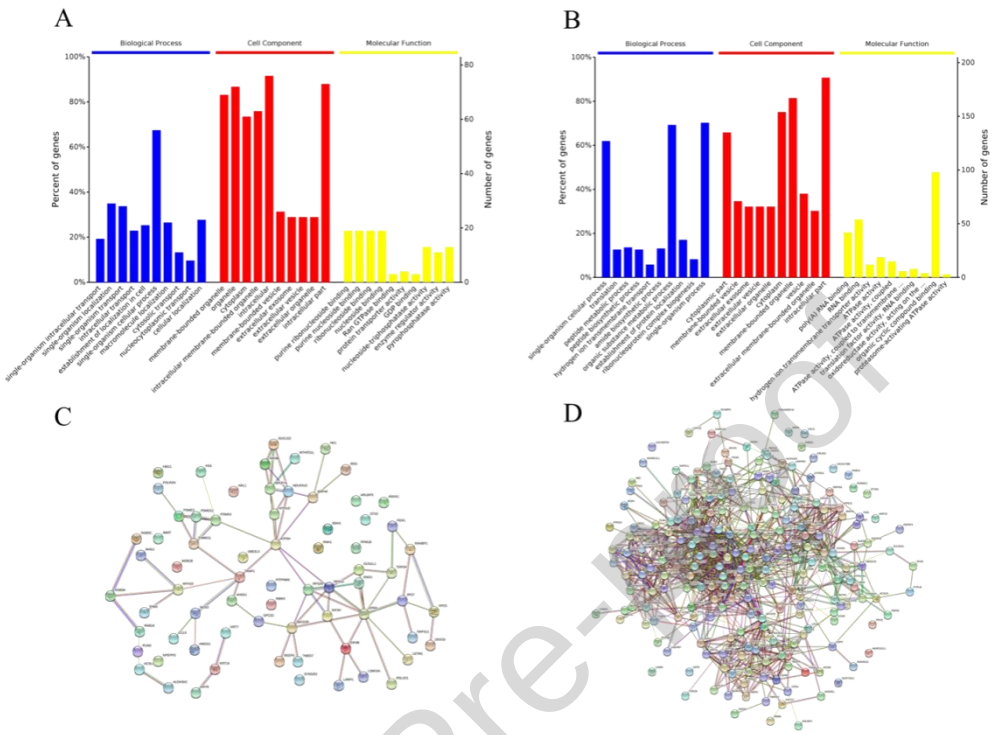


Figure 6



The authors declare that they have no conflict of interest.

Highlights

- Interferon-induced transmembrane proteins (IFITMs) are broad-spectrum antiviral factors. However, some viruses can use them to promote their own replication under certain conditions, such as HCoV-OC43 and SARS-CoV-2. In this study, we found that another coronavirus, Infectious Bronchitis Virus, can utilize chicken IFITM1 to promote its replication in specific cells. We try to study this phenomenon in terms of viral adsorption and invasion, protein subcellular localization, and interacting proteins. Our results investigate the relationship between coronavirus infection and host IFITM from a new perspective.