Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Chicken interferon-induced transmembrane protein 1 promotes replication of coronavirus infectious bronchitis virus in a cell-specific manner

Hao Li a,b,1, Ruiqi Ni a,b,1, Kailu Wang a,b, Yiming Tian c, Huilin Gong a,b, Wenjun Yan a,b, Yizhi Tang a,b, Changwei Lei a,b, Hongning Wang a,b, Xin Yang a,b,*

a Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu 610064, China
b Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, Chengdu 610064, China
c Key Laboratory of Microbiology and Parasitology of Education Department of Guizhou, School of Basic Medical Science, Guizhou Medical University, Guiyang, China

ARTICLE INFO
Keywords: IFITM IBV Chicken virus LMH Coronavirus

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.

1. Introduction

Interferon-induced transmembrane proteins (IFITMs) belong to a family of small transmembrane proteins. The IFITM family gene was first discovered in 1984 in neuroblastoma cells treated with interferon (IFN) (Friedman et al., 1984). 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 (Tanaka et al., 2005). 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 (Liao et al., 2019). 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 (Smith et al., 2013; Okuzaki et al., 2017). 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 (Hatano et al., 2008; Yang et al., 2018; Li et al., 2019). 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 (Blyth et al., 2016), 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) (Brass et al., 2009; Jiang et al., 2010; Huang et al., 2011; Shi et al., 2021a). However, studies on avian IFITMs are scarce. The IFITM1 and IFITM3 of chicken

* Corresponding author at: Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu 610064, China
E-mail address: yangxiao9822@scu.edu.cn (X. Yang).
1 Correspondence: the authors contribute equally.

https://doi.org/10.1016/j.vetmic.2022.109597
Received 29 June 2022; Received in revised form 6 October 2022; Accepted 26 October 2022
Available online 28 October 2022
0378-1135/© 2022 Elsevier B.V. All rights reserved.
and duck are known to restrict influenza virus, rabies virus (Smith et al., 2013; Brass et al., 2009), and avian tembusu virus (ATMUV) (Chen et al., 2017) infection in vitro. Transgenic chicks expressing ifitm1 have been demonstrated to restrict H5N1 influenza viruses (Rohaim et al., 2021). Recent studies have demonstrated that infection with IBV will increase the expression of IFITMs in chicks (Steyn et al., 2020).

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 (Zhao et al., 2019). Certain non-enveloped viruses, such as reovirus (Anafu et al., 2013) and foot-and-mouth disease virus (Xu et al., 2014), can be inhibited by IFITMs. In addition, certain viruses can hijack IFITMs to facilitate their infections, such as human coronavirus OC43 (HCoV-OC43) (Zhao et al., 2014) and human cytomegalovirus (HCMV) (Warren et al., 2014). 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 (Bozzo et al., 2021). Several studies have demonstrated that the antiviral potency of IFITM proteins varies among different cell types (Warren et al., 2014), suggesting that IFITMs can work with other cellular proteins to modulate viral infection (Zhao et al., 2019).

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) (Zhai et al., 2019) 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 (Wu et al., 2019). Viral titers were determined by a 50% tissue culture infective dose (TCID50) or median embryo lethal dose (EID50). Specific details of virus infection on LMH, DF-1, HD11, and Vero have been previously described (Guan et al., 2018; Han et al., 2017). 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). According to the manufacturer’s instructions.

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 DNA Kit (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 PrimeScript™ Reagent Kit (TaKaRa, Japan) to synthesize cDNA. Next, qRT-PCR was performed to analyze the expression of corresponding mRNAs (Primers IBV Beaudette forward: TGCTGCTAAGGGTGCTGACT, reverse: AGTGTCGCCATCCGGAAATC; IBV WT forward:CGCTCAATGGTCGTCAG, reverse:CATCATCGCTCCTTCGGCTT; IBV M41 forward: GTGTTGAGGAGTGTTCT, reverse: AGCCATTCTACGCTTATGCC; NDV-F48E9 forward: AAGAAGAACTGGCC, reverse: AGGCTTCC-TAGGCAAG; FADV forward: AGGTACATGAGGAGCCCGG, reverse: CTTTGGGAGAGTGGCTTGC). The Cha-mq Uni-versal SYBR qPCR Master Mix was purchased from Vazyme (Nanjing, China). Relative fold changes in gene expression were normalized against chGAPDH or huGAPDH using the 2-ΔΔCt 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 °C, 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 1–2 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% CO2 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 2–3 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 °C. 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 (GraphPad Software, La Jolla, CA, USA), and Student’s t-test was used for all statistical analyses. Data are presented as mean ± standard deviation (SD).

Fig. 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: TTGCTTGTATCAGTTGTT R: GCCATGGTTGTCACTGTCTATTG). (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 ×).
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 chIFITM 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 pcDNA3.1 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,
compared to the negative control group (Fig. 2C). As an enveloped virus, IBV primarily utilizes clathrin-mediated endocytosis (CME) for entry, viral particles move affecting the fusion of viral membranes with cellular membranes (Smith et al., 2019). To assess whether chIFITM1 functions in the early stages of IBV infection, viral mRNA levels were determined by RT-qPCR. 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 huIFITM 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 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 (Smith et al., 2019). 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 (Wang et al., 2019). 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 endolysosomal membranes. To confirm if different functions of chIFITMs in different cells are attributed to their subcellular localization, HD11 and LMH cells were transfected with chIFITM1/2/3 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 (Sun et al., 2020a). 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 C-terminal domain (CTD) domains (Liao et al., 2019). Among them, the component consisting of two TMDs and one CIL is defined as the CDD225 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. 5 A). 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 CIL), involved in the process of co-promoting IBV proliferation.
Fig. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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. 6 A-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 (Tay et al., 2012). 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 (Kawaguchi et al., 1987) 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 (Hornich et al., 2021). Similarly, the overexpression of IFITM1 and IFITM3 consistently enhanced HPV infectivity in several epithelial cell lines and keratinocytes (Warren et al., 2014). Furthermore, IFITM1 expression enhanced the KSHV, EBV, and HSV-2 infection of BJAB and HMVEC-d cells (Hussein and Akula, 2017).

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 (Diamond and Farzan, 2013). 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 (Smith et al., 2013; Zhao et al., 2021) 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 (Hornich et al., 2021; Bailey et al., 2014). Studies have demonstrated that endogenous IFITM can promote SARS-CoV-2 virus infection, whereas exogenously expressed IFITM exerts the opposite effect (Prelli Bozzo et al., 2021; Shi et al., 2021b). 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 (Smith et al., 2019; Sun et al., 2020a). 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 (Sun et al., 2020a). Sun’s study (Sun et al., 2020b) 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 (Wang et al., 2014). However, records of IFITM-interacting proteins are sparse. Maria et al (Gomez-Herranz et al., 2019) studied the interaction of proteins with IFITM1 using SWATH-IP mass spectrometry. We also assayed the

Fig. 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.
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 (Kelemen et al., 2021; Provance et al., 2021; Sakamoto et al., 2020). 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 (Liang et al., 2020). 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 (Kelemen et al., 2021; Provance et al., 2021; Sakamoto et al., 2020). 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 (Xu et al., 2020; Yount et al., 2010; Chesarino et al., 2014; Shan et al., 2013). It remains to be verified whether the modifications of IFITMs 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 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.

CRediT authorship contribution statement

Conceptualization: HL, RQL and XY. Methodology: HL, YMT and RQL. Validation: HL and RQL. Formal Analysis: RQL, HLG and KLB. Investigation: RQL, HLG and KLB. 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.

Funding

This research was supported by the Earmarked Fund for Modern Agroindustry Technology Research System (CARS-40-K14) and the Fundamental Research Funds for the Central Universities (grant no. SCU2021D006).

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.

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.

References

Friedman, R.L., Manly, S.P., McMahan, M., Kerr, I.M., Stark, G.R., 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell 38, 745–755.
Tanaka, S.S., Yamaguchi, Y.L., Tsui, B., Hickert, H., Yam, P.P.L., 2005. IFITM/mi Fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primate germ cell formation and resorption. Dev. Cell 9, 745–756.
Liao, Y., Goraya, M.U., Yuan, X., Zhang, B.G., Chiu, S.H., Chen, J.J., 2019. Functional involvement of interferon-inducible transmembrane proteins in antiviral immunity. Front. Microbiol. 10.
Smith, K.L., Gibson, M.S., Wang, R.S., Ferrara, F., Wright, E., Temperton, N., Kellam, P., Fife, M., 2013. Chicken interferon-inducible transmembrane protein 3 restricts influenza viruses and lyssaviruses in vitro. J. Virol. 87, 12957–12966.
Okunski, Y., Kidani, S., Kanesaka, H., Iijima, S., Nishijima, K., 2017. Characterization of chicken interferon-inducible transmembrane protein 10. Biosci. Biotechn. Bioch. 81, 914–921.
Hatano, H., Kudo, Y., Ogawa, I., Tsunematsu, T., Kikuchi, A., Abiko, Y., Takata, T., 2020. IFN-induced transmembrane protein 1 promotes invasion at early stage of head and neck cancer progression. Clin. Cancer Res. 16, 6097–6105.
Yang, J., Li, L., Xi, Y., Sun, R.M., Wang, H., Ren, Y.X., Zhao, L.F., Wang, X.L., Li, X.J., 2018. Combination of IFITM1 knockdown and radiotherapy inhibits the growth of glioblastoma. Cancer Sci. 111, 3115–3126.
Li, J.Z., Chen, N., Gong, X.B., 2019. Prognostic implications of aberrantly expressed methyltransferase-driven genes in hepatocellular carcinoma: a study based on the cancer genome atlas. Mol. Med. Rep. 20, 5304–5314.
Blyth, G.A.D., Chan, W.F., Webster, R.G., Mager, K.E., 2016. Duck interferon-inducible transmembrane protein 3 mediates restriction of influenza viruses. J. Virol. 90, 103–116.
Brass, A.L., Huang, I.C., Benita, Y., John, S.P., Krishnan, M.N., Feeley, E.M., Ryan, B.J., Weyer, J.L., van de Werken, L., Ladrang, E., Adams, D.J., Hahn, R.S., Varani, D., Elledge, S.J., 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, west Nile virus, and dengue viruses. Cell 139, 1243–1254.
Jiang, D., Wiedmer, J.M., Qing, M., Pan, X.B., Guo, H.T., Xu, C.X., Zhang, X.C., Birx, A., Chang, L.H., Shi, P.Y., Block, T.M., Guo, J.T., 2010. Identification of five interferon-induced cellular proteins that inhibit west Nile virus and dengue viruses. J. Virol. 84, 8332–8341.
Huang, I.C., Bailey, C.C., Weyer, J.L., Radiositsky, S.R., Becker, M.M., Shiang, J.J., Benita, Y., Ahmed, A.A., Chu, X.Y., Liang, L.A., Longobardi, L.F., Bolz, D., Kuhn, J.H., Elledge, S.J., Bavarai, S., Denison, M.R., Choe, H., Farzan, M., 2011. Distinct patterns of IFITM-mediated restriction of flaviviruses, SARS coronavirus, and influenza virus A. Plos Path. 7, 1–9.
Shi, G.L., Kenney, A.D., Kudryashova, L., Zani, A., Zhang, L.Z., Lai, K.K., Hall-Stoodley, L., Robinson, R.T., Kudryashov, D.S., Compton, A.A., Yount, J.S., 2021a. Opposing activities of IFITM proteins in SARS-CoV-2 infection. Embo J. 40, 5750–5765.
Chen, S.X., Wang, L., Chen, J.Y., Zhang, L.L., Wang, S., Goraya, M.U., Chi, X.J., Na, Y., Shao, W.H., Yang, Z., Zeng, X.C., Chen, S.Y., Chen, J.J., 2017. Avian interferon-inducible transmembrane protein family effectively restricts avian tennis virus infection. Front Microbiol. 8.
Rohaim, M.A., Al-Nature, M.A., Abdelalabour, M.A., El Naggar, R.F., Madbouly, Y.M., Ahmed, K.A., Munir, M., 2021. Transgenic chicks expressing interferon-inducible transmembrane protein 1 (IFITM1) restrict highly pathogenic H5N1 influenza viruses. Int. J. Mol. Sci. 22, 13375–13390.
Shi, G.L., Kenney, A.D., Kudryashova, L., Zani, A., Zhang, L.Z., Lai, K.K., Hall-Stoodley, L., Robinson, R.T., Kudryashov, D.S., Compton, A.A., Yount, J.S., 2021b. Opposing activities of IFITM proteins in SARS-CoV-2 infection. Embo J. 40, 5750–5765.
Chen, S.X., Wang, L., Chen, J.Y., Zhang, L.L., Wang, S., Goraya, M.U., Chi, X.J., Na, Y., Shao, W.H., Yang, Z., Zeng, X.C., Chen, S.Y., Chen, J.J., 2017. Avian interferon-inducible transmembrane protein family effectively restricts avian tennis virus infection. Front Microbiol. 8.
Hosseini, M., Pagh, M., Blom, L., 2008. Functional analysis of the interferon inducible transmembrane proteins in influenza A virus infection. Front Immunol. 4.
Ahmed, K.A., Munir, M., 2021. Transgenic chicks expressing interferon-inducible transmembrane protein 1 (IFITM1) restrict highly pathogenic H5N1 influenza viruses. Int. J. Mol. Sci. 22, 13375–13390.
Shi, G.L., Kenney, A.D., Kudryashova, L., Zani, A., Zhang, L.Z., Lai, K.K., Hall-Stoodley, L., Robinson, R.T., Kudryashov, D.S., Compton, A.A., Yount, J.S., 2021a. Opposing activities of IFITM proteins in SARS-CoV-2 infection. Embo J. 40, 5750–5765.
Chen, S.X., Wang, L., Chen, J.Y., Zhang, L.L., Wang, S., Goraya, M.U., Chi, X.J., Na, Y., Shao, W.H., Yang, Z., Zeng, X.C., Chen, S.Y., Chen, J.J., 2017. Avian interferon-inducible transmembrane protein family effectively restricts avian tennis virus infection. Front Microbiol. 8.
particles bearing antigens of infectious bronchitis virus and Newcastle disease virus. Virus - Basel 11.
Guo, P., Tian, Y.M., Han, X.X., Yang, X., Wang, H.N., 2018. Complete genome sequence and pathogenicity of fowl adenovirus serotype 4 involved in hydropneumonia syndrome in Southwest China. Micro Pathog. 117, 290–296.
Han, X.X., Tian, Y.M., Guo, R., Gao, W.Q., Yang, X., Zhou, L., Wang, H.N., 2017. Infectious bronchitis virus infection induces apoptosis during replication in chicken macrophage HD11 cells. Virus - Basel 9.
Smith, S.E., Busse, D.C., Blüter, S., Weston, S., Soria, C.D., Laksono, B.M., Clare, S., Van Nieuwenhooft, S., Van den Hoogen, B.G., Clement, M., Marsden, M., Humphreys, I.R., Marsh, M., de Swart, R.I., Wath, R.S., Tregoning, J.S., Kellam, P., 2019. Interferon-induced transmembrane protein 1 restricts replication of viruses that enter cells via the plasma membrane. J. Virol. 93.
Wang, H., Yuan, X., Sun, Y.J., Man, X., Meng, C.C., Tan, L., Song, C.P., Qiu, X.S., Ding, C., Liao, Y., 2019. Infectious bronchitis virus entry mainly depends on clathrin mediated endocytosis and requires classical endosomal/lysosomal system. Virology 528, 118–136.
Sun, F., Xia, Z.Q., Han, Y.W., Gao, M.J., Wang, L.Y., Wu, Y.L., Sabatier, J.M., Miao, L.X., Cao, Z.J., 2020a. Topology, antiviral functional residues and mechanism of IFITM1. Virus - Basel 12.
Tay, F.P.L., Huang, M., Wang, L., Yamada, Y., Liu, D.X., 2012. 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, 421–430.
Kawahuchi, T., Nomura, K., Hirayama, Y., Kitagawa, T., 1987. Establishment and characterization of a chicken hepatocellular-carcinoma cell-line, Lmh. Cancer Res 47, 4460–4464.
Hornich, B.F., Grosskopf, A.K., Dcosta, C.J., Schlagowski, S., Hahn, A.S., 2021. KSHV-induced persistent infection in human lung cancer. Int J. Mol. Sci. 21.
Prelli Bozzo, C., Nchioua, R., Volcic, M., Koepke, L., Kruger, J., Schutz, D., Heller, S., Sturzel, C.M., Kniec, D., Conzelmann, C., Muller, J., Zech, F., Braun, E., Gross, R., Wettstein, L., Weil, T., Weiss, J., D’iofano, F., Rodriguez Alfonso, A.A., Wiese, S., Sauter, D., Munch, J., Goffinet, C., Catanesi, A., Schon, M., Boeckers, T.M., Stenger, S., Sato, K., Just, S., Kleger, A., Sparrer, K.M.J., Kirchhoff, F., 2021. IFITM proteins promote SARS-CoV-2 infection and are targets for virus inhibition in vitro. Nat. Commun. 12, 4584.
Shi, G., Kenney, A.D., Kudryashova, E., Zani, A., Zhang, L., Lai, K.K., Hall-Stoodley, L., Robinson, R.T., Kudryashov, D.S., Compton, A.A., Yount, J.S., 2021b. Opposing activities of IFITM proteins in SARS-CoV-2 infection. EMBO J. 40, e106551.
Sun, F., Xia, Z., Han, Y., Gao, M., Wang, L., Wu, Y., Sabatier, J.M., Miao, L., Cao, Z., 2020b. Topology, antiviral functional residues and mechanism of IFITM1. Viruses 12.
Wang, S., Chi, X.J., Wei, H.T., Chen, Y.H., Chen, Z.L., Huang, S.L., Chen, J.L., 2014. Influenza virus-induced degradation of eukaryotic translation initiation factor 4B contributes to viral replication by suppressing IFITM3 protein expression. J. Virol. 88, 8375–8385.
Gomez-Herranz, M., Nekulova, M., Faktor, J., Hrynchova, L., Kote, S., Sinclair, E.H., Nenutil, R., Vojtesek, B., Ball, K.L., Hupp, T.R., 2019. The effects of IFITM1 and IFITM3 gene deletion on IFN gamma stimulated protein synthesis. Cell Signal 60, 39–56.
Kelemen, A., Carmi, I., Ozsvold, A., Lorincz, P., Petovari, G., Tolgyes, T., Dede, K., Bursics, A., Buzas, E.I., Wiener, Z., 2021. IFITM1 expression determines extracellular vesicle uptake in colorectal cancer. Cell Mol. Life Sci. 78, 7009–7024.
Provonce, O.K., Geanes, E.S., Lui, A.J., Roy, A., Holloran, S.M., Gunewardena, S., Hagan, C.R., Weir, S., Lewis-Wambi, J., 2021. Disrupting interferon-alpha and NF-kappaB crosstalk suppresses IFITM1 expression attenuating triple-negative breast cancer progression. Cancer Lett. 514, 12–29.
Sakamoto, S., Inoue, H., Kohda, Y., Ohba, S., Mizutani, T., Kawada, M., 2020. Interferon-induced transmembrane protein 1 (IFITM1) promotes distant metastasis of small cell lung cancer. Int J. Mol. Sci. 21.
Jiang, R.B., Li, X.X., Zhu, X.D., 2020. Deciphering the roles of IFITM1 in tumors. Mol. Diagn. Ther. 24, 453–441.
Xu, Z., Li, X.L., Xue, J.C., Shen, L.Y., Zheng, W.M., Yin, S.G., Xu, J., 2020. S-palmitoylation of swine interferon-inducible transmembrane protein is essential for its anti-JEV activity. Virology 548, 82–92.
Yount, J.S., Moleda, B., Yang, Y.Y., Charron, G., Moran, T.M., Lopez, C.B., Hang, H.C., 2010. Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. Nat. Chem. Biol. 6, 610–614.
Chesarino, N.M., McMichael, T.M., Hath, J.C., Yount, J.S., 2014. Phosphorylation of the antiviral protein interferon-inducible transmembrane protein 2 (IFITM3) dually regulates its endocytosis and ubiquitination. J. Biol. Chem. 289, 11986–11992.
Shan, Z., Han, Q.L., Nie, J., Cao, X.Z., Chen, Z.J., Yin, S.Y., Gao, Y.Y., Lin, F., Zhou, X.H., Xu, K., Fan, H.M., Qian, Z.K., Sun, B., Zhong, J., Li, B., Tsun, A., 2013. Negative Regulation of Interferon-induced Transmembrane Protein 3 by SEIT-mediated Lysine Monomethylation. J. Biol. Chem. 288, 35093–35103.
Moller, S., Croning, M.D.R., Apweiler, R., 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17, 646–653.