Duck Interferon-Inducible Transmembrane Protein 3 Mediates Restriction of Influenza Viruses

Graham A. D. Blyth,^a Wing Fuk Chan,^a Robert G. Webster,^b Katharine E. Magor^a

Department of Biological Sciences and Li Ka Shing Institute of Virology, University of Alberta, Edmonton, Alberta, Canada^a; Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA^b

ABSTRACT

Interferon-inducible transmembrane proteins (IFITMs) can restrict the entry of a wide range of viruses. IFITM3 localizes to endosomes and can potentially restrict the replication of influenza A viruses (IAV) and several other viruses that also enter host cells through the endocytic pathway. Here, we investigate whether IFITMs are involved in protection in ducks, the natural host of influenza virus. We identify and sequence duck *IFITM1*, *IFITM2*, *IFITM3*, and *IFITM5*. Using quantitative PCR (qPCR), we demonstrate the upregulation of these genes in lung tissue in response to highly pathogenic IAV infection by 400-fold, 30-fold, 30-fold, and 5-fold, respectively. We express each IFITM in chicken DF-1 cells and show duck IFITM1 localizes to the cell surface, while IFITM3 localizes to LAMP1-containing compartments. DF-1 cells stably expressing duck IFITM3 (but not IFITM1 or IFITM2) show increased restriction of replication of H1N1, H6N2, and H11N9 IAV strains but not vesicular stomatitis virus. Although duck and human IFITM3 share only 38% identity, critical residues for viral restriction are conserved. We generate chimeric and mutant IFITM3 proteins and show duck IFITM3 does not require its N-terminal domain for endosomal localization or antiviral function; however, this N-terminal end confers endosomal localization and antiviral function on IFITM1. In contrast to mammalian IFITM3, the conserved YXX θ endocytosis signal sequence in the N-terminal domain of duck IFITM3 is not essential for correct endosomal localization. Despite significant structural and amino acid divergence, presumably due to host-virus coevolution, duck IFITM3 is functional against IAV.

IMPORTANCE

Immune IFITM genes are poorly conserved across species, suggesting that selective pressure from host-specific viruses has driven this divergence. We wondered whether coevolution between viruses and their natural host would result in the evasion of IFITM restriction. Ducks are the natural host of avian influenza A viruses and display few or no disease symptoms upon infection with most strains, including highly pathogenic avian influenza. We have characterized the duck IFITM locus and identified IFITM3 as an important restrictor of several influenza A viruses, including avian strains. With only 38% amino acid identity to human IFITM3, duck IFITM3 possesses antiviral function against influenza virus. Thus, despite long coevolution of virus and host effectors in the natural host, influenza virus evasion of IFITM3 restriction in ducks is not apparent.

Type I interferons are produced in response to viral infection and rapidly upregulate hundreds of interferon-stimulated genes (ISGs) in surrounding cells to induce an antiviral state. The interferon-inducible transmembrane proteins (IFITMs) are recently recognized ISGs with antiviral activity against a broad range of viruses (1, 2). Originally identified in a cDNA screen of genes upregulated by type I interferons, their antiviral properties have since been well characterized *in vitro* (3, 4). IFITMs inhibit the entry of several pathogenic viruses, including influenza A virus (IAV), West Nile virus, dengue virus, severe acute respiratory syndrome (SARS) coronavirus, filoviruses, vesicular stomatitis virus (VSV), and hepatitis C virus (HCV), among others (5–8).

IFITM1 is expressed predominately at the cell surface and in early endosomes (6), consistent with an ability to restrict viral pathogens that enter host cells at the plasma membrane or early endosomes (8, 9). IFITM2 and IFITM3 localize to late endosomes and lysosomes, where they preferentially restrict viruses that utilize the endocytic pathway to invade host cells (6, 10, 11). The N-terminal domain of IFITM3 contains a YXX θ endocytosis motif that is required for correct cellular localization (12, 13). Mutation of the critical tyrosine residue within this sequence is sufficient to achieve a loss of association with endosomes, resulting in the accumulation of IFITM3 at the cell surface while at the same

time abolishing antiviral function against influenza viruses (13). Palmitoylation on cysteine residues in IFITM3 increases membrane clustering and is necessary for complete antiviral activity (11). Ubiquitination at conserved lysine residues (14) and tyrosine phosphorylation (15) also have been identified as contributors to IFITM3 cellular localization and antiviral function. IFITM1 can exist in intracellular compartments; however, in contrast to IFITM3, it uses a noncanonical C-terminal dibasic signal sequence to localize to intracellular compartments (16, 17).

IFITM3 has a well-defined role in the restriction of influenza A virus (IAV). *In vitro*, IFITM3 alone is responsible for between 40 and 70% of the antiviral activity of type I interferon against IAV (5). *IFITM3del* mice display severe morbidity and mortality after

Received 25 June 2015 Accepted 30 September 2015

Accepted manuscript posted online 14 October 2015

Citation Blyth GAD, Chan WF, Webster RG, Magor KE. 2016. Duck interferon-inducible transmembrane protein 3 mediates restriction of influenza viruses. *J Virol* 90:103–116. doi:10.1128/JVI.01593-15.

Editor: R. M. Sandri-Goldin

Address correspondence to Katharine E. Magor, kmagor@ualberta.ca.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

infection with low-pathogenicity IAV *in vivo* (18, 19). Previous reports also identified an enrichment of the rs12252-C allele of IFITM3 in patients hospitalized with seasonal or pandemic influenza infections and in severe influenza virus infections in Han Chinese patients (19, 20). Collectively, these results demonstrate that IFITM3 is a significant contributor to the innate immune defense against influenza viruses and is an important factor in the outcome of an influenza virus infection.

Arenaviruses and several DNA viruses, such as human papillomavirus, cytomegalovirus, and adenovirus, are resistant to IFITM restriction (5, 21). HIV can evolve to escape from IFITM1 restriction, which suppresses HIV replication but not entry, remarkably involving a single mutation in the Env gene in the CD4 binding site and truncation of the Vpu gene (22). In fact, some viral pathogens, such as human coronavirus OC43 and human papillomavirus 16 (HPV16), use IFITMs to promote their own infection (21, 23). Intriguingly, these are human-adapted viruses in human hosts, suggesting mechanisms to evade IFITM function arise in the natural host.

Influenza A viruses circulate globally primarily using their natural host, wild waterfowl. The natural host may be infected with all strains of IAV with little to no disease symptoms and can transmit the virus to agriculturally important species, such as chickens or swine, or to humans, where sporadic infections can cause high rates of morbidity and mortality (24, 25). While of critical importance, especially for zoonotic pathogens, the immune response to viruses in their reservoir species is rarely studied (26). We study the innate immune response to influenza virus in the natural host and have shown that ducks greatly upregulate ISGs following infection with highly pathogenic IAV (27, 28), and we identified an IFITM gene (28). Here, we characterize the IFITM genes of White Pekin ducks and their expression upon influenza virus infection. We show dIFITM3 is a potent restrictor of IAV replication in avian cells, including avian strains. In addition, we demonstrate that the N-terminal YXX θ endocytic signal sequence of dIFITM3 is not solely responsible for endosomal localization or antiviral function.

MATERIALS AND METHODS

Identification, sequencing, and analysis of duck IFITMs. Partial sequences of duck IFITM1, IFITM2, IFITM3, and IFITM5 were obtained through analysis of scaffold 2493 of the mallard duck (*Anas platyrhynchos*) genome (ENSEMBL, BGI_duck_1.0). Genes flanking the IFITM locus, *B4GALNT4* and *ATHL1*, were identified and the genomic region in between analyzed. To obtain exon 1 sequences, 5' rapid amplification of cDNA ends was used for IFITM3 (Clontech), and RNA transcriptome sequences were used to obtain IFITM2. Full-length duck IFITM coding sequences were amplified from cDNA from duck lung tissue with Phusion high-fidelity DNA polymerase (New England BioLabs) using primers that incorporate NheI and NotI restriction enzyme sites. PCR products were cloned into pCR2.1-TOPO (Invitrogen) and sequenced in the forward and reverse directions using BigDye Terminator v3.1 (Applied Biosystems). Sequences were analyzed using ContigExpress (Invitrogen). Each product subsequently was cloned to incorporate an N-terminal V5 tag into the expression vector pcDNA3.1/Hygro+ (Invitrogen). Sequence alignment of IFITMs was performed using T-COFFEE in JalView (29). A maximum likelihood tree of IFITMs was generated using PhyML with 100 bootstrap replications (30).

Quantification of IFITM expression by real-time PCR. Infection of White Pekin ducks with H5N1 A/Vietnam/1203/04 (VN1203) and H5N2 A/mallard/BC500/05 (BC500) and generation of cDNA were described previously (27). Titers of oropharyngeal swabs for VN1203 ranged from 10^2 to 10^4 , and those from cloacal swabs for BC500 were from 10^5 to 10^7 ,

TABLE 1 Primer and probe sequences for qPCR analysis of duck and chicken IFITM gene expression

Gene	Primer sequences ^a (5'→3')
<i>GAPDH</i>	
Primer 1	AAATTGTCAGCAATGCCTCTTG
Primer 2	TGGCATGGACAGTGGTCATAA
Probe	56-FAM-ACCACCAAC-ZEN-TGCCTGGCGCC-3IABkFQ
<i>dIFITM1</i>	
Primer 1	CTTTGTGCTCTGGTCTTTC
Primer 2	GTTGCCAGTGTCTATC
Probe	56-FAM-CGCCATGTA-ZEN-CTGCAACCCCTTCT-3IABkFQ
<i>dIFITM2</i>	
Primer 1	TTCAACGTCCTGATCGGTTAC
Primer 2	GATGTTCAACACCTTGGCC
Probe	56-FAM-TCTGCTTCC-ZEN-CCGCGCTCAT-3IABkFQ
<i>dIFITM3</i>	
Primer 1	CACCGCCAAGTACCTGAACA
Primer 2	CGATCAGGGCGATGATGAG
Probe	56-FAM-CACGGCCCT-ZEN-GCTGCTCAACATCT-3IABkFQ
<i>dIFITM5</i>	
Primer 1	TCCACCTCTCCAAGCTC
Primer 2	GCTTGTGTTGAAGTGGTAG
Probe	56-FAM-CCAGGACTC-ZEN-CGTGGCCTTTTCA-3IABkFQ
<i>chIFITM1</i>	
Primer 1	TGTTAAAGATCTTCGCTGTCCTC
Primer 2	TCTGGTCCCTCTCAACTTTG
Probe	56-FAM-CCTTCTGCC-ZEN-TGGGCTTATGTGCT-3IABkFQ
<i>chIFITM2</i>	
Primer 1	GCTTCCATACCGCCGAG
Primer 2	TCATCCTCTGGTCCCTCTTC
Probe	56-FAM-CTTCTGCTT-ZEN-CCCCTCGCTCATCTT-3IABkFQ
<i>chIFITM3</i>	
Primer 1	TTGATCAGATGGGCCGTG
Primer 2	GCTGTACGCCAACGTCTG
Probe	56-FAM-AGGGATCGC-ZEN-AAAGTCCTGGGTG-3IABkFQ

^a FAM, 6-carboxyfluorescein; ZEN, internal quencher; 3IABkFQ, 3' Iowa black fluorescent quencher.

as reported previously (28). All animal experiments in that study were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and performed in compliance with relevant institutional policies, National Institutes of Health regulations, and the Animal Welfare Act. Primers and probes were generated (IDT Technologies) and validated for linear amplification of each duck IFITM and compared to the *GAPDH* endogenous control (Table 1). Changes in target gene expression are relative to those of a mock-infected animal. Analysis was performed using relative quantification of gene expression ($\Delta\Delta C_T$, where C_T is threshold cycle) using 7500 Fast System software v1.4 (Applied Biosystems) as previously described (28). Analysis of chicken and duck IFITM expression in stably transfected DF-1 cells was performed using qPCR, analyzing the expression of each overexpressed duck IFITM relative to that of its respective chicken IFITM. The specificity of primers and probes was confirmed against a panel of cloned IFITM genes.

Generation of chimeric proteins and point mutants. Chimeric proteins swapping the N-terminal domains of dIFITM1 and dIFITM3 were generated by overlap extension PCR. Briefly, the N-terminal regions of dIFITM1 and dIFITM3 were amplified using forward primers previously described and reverse primers containing a 5' overhang that corresponds to the CD225 domain of either dIFITM1 or dIFITM3. CD225 and the C-terminal tail were amplified for dIFITM1 and dIFITM3 using the reverse primers previously described and a forward primer containing a 5' overhang that corresponds to the N-terminal region of either dIFITM1 or

dIFITM3. PCR products of the N terminus of dIFITM1 and the CD225 domain of dIFITM3 were combined, PCR products of the N-terminal region of dIFITM3 and the CD225 domain of dIFITM1 were combined, and a final PCR was performed to create full-length chimeric proteins using forward and reverse primers. Point mutants of dIFITM3 were generated by PCR using site-directed mutagenesis. All chimeric proteins and point mutants were cloned into pcDNA3.1/Hygro⁺, and sequences were confirmed.

Cell culture, transfections, and generation of stable cell lines expressing IFITMs. DF-1 chicken fibroblasts, HeLa cells, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma). For transient transfections, cells were seeded overnight in 96-well plates (3×10^4 cells) and 24 h later were transfected with 0.2 μ g of plasmid DNA/well using 0.5 μ l of Lipofectamine 2000 (Invitrogen). Infections were performed 24 h after transfection. Stably expressing DF-1 cells were generated by seeding cells overnight in 6-well plates (8×10^5 cells) and 24 h later were transfected with 1.25 μ g of linearized plasmid DNA/well using 3.75 μ l of Lipofectamine 2000. Forty-eight hours after transfection, cells were put under selection using hygromycin and surviving cells were expanded. Individual clones were isolated by limiting dilution and expression of IFITM proteins in clones screened by Western blotting.

Western blotting. Whole-cell lysates of DF-1 cells were collected using lysis buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% [vol/vol] Triton X-100) with cOmplete Mini, EDTA-free proteinase inhibitor (Roche Diagnostics). Cell lysates were boiled in $1 \times$ Laemmli buffer before separation by SDS-PAGE and transfer to a nitrocellulose membrane. Western blotting was performed using mouse anti-V5 antibody (Life Technologies) at 1:5,000 and goat anti-mouse horseradish peroxidase (HRP) at 1:5,000 (Bio-Rad). Proteins were visualized by chemiluminescence using the ECL kit (GE Healthcare).

Cell culture and virus infections. Twenty-four hours after transfection or plating of stably expressing DF-1 cells, cells were challenged with A/chicken/California/431/00 (H6N2), A/duck/Memphis/546/1974 (H11N9), A/Puerto Rico/8/1934 (H1N1), or recombinant VSV-green fluorescent protein (rVSV-GFP) at the indicated multiplicity of infection (MOI). For influenza viruses, cells were cultured in DMEM supplemented with 0.3% bovine serum albumin (BSA) and L-(tosyl-amido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (0.1 μ g/ml) (Worthington Biochemical). Cells were challenged with influenza A virus for 30 min or rVSV-GFP for 1 h before unbound virus was removed. Fresh medium was added, and cells were incubated for 6 h (influenza virus) or 12 h (rVSV-GFP) before fixing.

Fluorescence microscopy analysis of viral infection. Six hours after infection with influenza A virus, cells were fixed in 1% formaldehyde and washed with 0.1% Triton X-100 for 10 min. Cells were washed three times with $1 \times$ PBS and blocked for 1 h with 4% BSA. The cells then were stained with anti-nucleoprotein-fluorescein isothiocyanate (FITC) (Argene) for 1 h, followed by staining with Hoechst 33342 (Life Technologies). Images were taken with the Operetta high-content imaging system (PerkinElmer) to determine the percentage of infected cells.

Flow-cytometric analysis of viral infection. Infected DF-1 cells were harvested using 0.25% Trypsin-EDTA (Invitrogen) and fixed in 1% paraformaldehyde. Influenza virus-infected cells were washed for 10 min with 0.1% Triton-X in PBS before staining with anti-nucleoprotein-FITC (Argene). rVSV-GFP-infected cells were visualized directly. The percentage of FITC- or GFP-positive cells was determined by flow cytometry.

Plaque assays. Supernatant from infected DF-1 cells was collected 12 h postinfection and serially diluted in infection media. Monolayers of MDCK cells grown in 6-well plates were infected with serially diluted supernatants. After 1 h, supernatants were removed and cells were washed with PBS. Cells then were overlaid with infection media containing 0.9% agar. After 72 h cells were stained with 0.1% crystal violet solution to calculate the number of PFU.

Cellular localization of IFITMs by confocal microscopy. DF-1, HeLa, or HEK293T cells were seeded onto coverslips in 6-well plates at

8×10^5 cells, and the cells were transfected with 1.5 μ g dIFITM constructs. Twenty-four hours after transfection, cells were fixed in ice-cold 100% methanol (DF-1 cells) or 1% PFA (HeLa and HEK293T cells) for 20 min and blocked in 4% BSA for 1 h. The cellular location of each duck IFITM was determined by staining with rabbit anti-V5 conjugated to Dylight650 (ab117489; Abcam). Chicken endosomes were stained using a mouse anti-LEP100 antibody (LEP100 IgG; Developmental Studies Hybridoma Bank) followed by staining with a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (A-11001; Thermo Scientific). The nuclei of cells were stained with Hoechst 33342 (Life Technologies). Images were taken on a Zeiss LSM 510 confocal microscope. Colocalization analysis of the Pearson's coefficient between chicken LAMP1 and each V5-tagged protein was completed using ImageJ (31).

RESULTS

Characterization of the duck IFITM repertoire. Previously, using subtractive suppressive hybridization, we identified a duck *IFITM* gene as an innate immune gene expressed early in duck lung tissue in response to infection with highly pathogenic influenza A virus (28). The potential involvement of IFITMs in duck lung tissue in response to IAV led us to characterize the duck *IFITM* gene repertoire. Analysis of the putative duck *IFITM* locus on scaffold 2493 revealed two predicted IFITM genes and the two flanking genes, *B4GALNT4* and *ATHL1*. Using gene synteny from the chicken *IFITM* locus (32) and transcripts from transcriptome sequencing (RNA-seq) (33), we identified the corresponding duck homologues *dIFITM5*, *dIFITM2*, *dIFITM1*, and *dIFITM3* (Fig. 1A). *dIFITM1*, *dIFITM2*, *dIFITM3*, and *dIFITM5* are all composed of two exons and contain a CD225 domain, which is characteristic of *IFITM* genes. Transcripts corresponding to each gene were amplified from cDNA and amino acid sequences aligned with their avian and mammalian homologues (Fig. 1B). The immune-related duck IFITMs, *dIFITM1*, *dIFITM2*, and *dIFITM3*, have 41%, 68%, and 76% amino acid identity to the chicken orthologs, respectively, whereas the predicted nonimmune *dIFITM5* has 91% identity. The CD225 domain is the most highly conserved region of IFITMs, as the CD225 domain of *dIFITM3* has 96% and 63% identity to CD225 of chicken and human IFITM3, respectively. Hydrophobic transmembrane regions are highly conserved in all IFITMs, as are cysteine residues that are palmitoylated in all IFITMs tested (11, 34). Duck IFITM1 has a unique insertion in exon 1 not seen in chicken IFITM1. The dibasic residues, implicated in the localization of human IFITM1 (16), are not conserved in ducks. Several residues shown to be important for antiviral function of human IFITM3 are conserved in ducks, including an endosomal localization signal at Y20 (12, 13), C71 and C72, which undergo palmitoylation (11, 14), lysines K83, K88, and K104, which are sites of ubiquitination (14), and tyrosine Y99, which influences antiviral activity against influenza virus (35). Significant divergence between avian and human IFITM1 and IFITM2 suggests they are not direct orthologues, while avian and human IFITM3 and IFITM5 share critical residues and greater conservation. Phylogenetic analysis of the relationship between duck, chicken, human, and mouse IFITM proteins reveals that duck IFITMs group most closely to their respective chicken orthologues (Fig. 1C). The orthology to human genes is uncertain because the mammalian IFITM1, IFITM2, and IFITM3 homologues segregate to the same clade. Furthermore, the human *IFITM1* and *IFITM2* genes are in a transcriptional orientation that is the opposite of that of duck or chicken (32, 36). Therefore, we have

FIG 1 Organization of duck IFITM locus with sequence alignment and maximum likelihood tree of duck, chicken, human, and mouse IFITMs. (A) The duck IFITM genes between *ATHL1* and *B4GALNT4* were annotated using gene synteny from the chicken IFITM locus. (B) IFITM sequences were aligned using T-COFFEE. Membrane-spanning domains are underlined in each sequence alignment. The noncanonical dibasic signal sequence in IFITM1 is indicated with a double arrow. Important residues for IFITM3 function are noted. The conserved YXX θ motif is boxed. Symbols: *, palmitoylated cysteine residues; Δ , ubiquitinated lysine residues. (C) A maximum-likelihood tree was generated using 100 bootstrap values to show similarity of orthologous IFITMs.

identified four duck *IFITM* genes in the same order and orientation as those of the chicken orthologues.

Duck IFITMs are upregulated in response to influenza A virus infection. Previously, analysis of the duck transcriptome

showed transcripts of IFITM3, IFITM5, and IFITM10 were increased in duck lung tissue after infection with two different H5N1 strains, A/duck/Hubei/49/05 and A/goose/Hubei/65/05 (33). To determine the extent of upregulation of duck IFITMs in response

FIG 2 Duck IFITMs are upregulated in lung tissue in response to highly pathogenic IAV infection. Total RNA was isolated from duck lung tissue 1 and 3 days postinfection with PBS (mock), BC500 (low-pathogenicity IAV), or VN1203 (highly pathogenic IAV). IFITM1 (A), IFITM2 (B), IFITM3 (C), and IFITM5 (D) expression was measured using qPCR and compared to that of the mock-infected group.

to infection with IAV, we examined their relative expression levels using qPCR on cDNA from lung tissue from ducks treated with PBS (mock infection), infected with low-pathogenicity avian H5N2 strain A/British Columbia 500/05 (BC500), or infected with highly pathogenic H5N1 strain A/Vietnam1203/04 (VN1203) (Fig. 2). *dIFITM1* was highly upregulated by 370-fold at 1 day postinfection (dpi) and remained upregulated 406-fold at 3 dpi with VN1203 (Fig. 2A). *dIFITM1* also was upregulated 43-fold and 11-fold at 1 and 3 dpi with BC500, respectively (Fig. 2A). In addition, *dIFITM2* and *dIFITM3* were upregulated at 1 dpi with VN1203 24-fold and 28-fold, respectively (Fig. 2B and C). The expression of both *dIFITM2* and *dIFITM3* was only modestly upregulated 4-fold at 3 dpi with VN1203 and 2-fold and 3-fold at 1 dpi with BC500, respectively (Fig. 2B and C). Interestingly, *dIFITM5*, which has been characterized as a bone-specific gene in mammalian species, was upregulated 5-fold at 1 dpi with VN1203 (Fig. 2D). We observed 2-fold upregulation of *dIFITM1* and no upregulation of *dIFITM3* and *dIFITM5* in intestinal tissue at 1 dpi with BC500 (data not shown). We noted higher basal expression of *dIFITM1* in intestine than in lung from mock-infected animals, as previously observed in chicken (32). Thus, expression of *IFITM1*, *IFITM2*, and *IFITM3* was upregulated in the lung, the site of infection with the highly pathogenic VN1203, but was not upregulated in the intestine, the replication site for low-pathogenicity strain BC500.

Duck IFITM3 restricts influenza A viruses. Given the antiviral function of orthologous IFITMs, we examined whether duck

IFITMs could inhibit IAV replication in DF-1 cells. We performed an initial screen of the antiviral properties of three duck IFITMs using transient transfection of constructs expressing N-terminal V5-tagged IFITM proteins in DF-1 chicken embryonic fibroblast cells. We challenged DF-1 cells overexpressing *dIFITMs* with either H6N2 or H11N9 at an MOI of 1 or 5, and we determined the percentage of infected cells expressing influenza NP protein by high-content fluorescence microscopy. The overexpression of *dIFITM3* reduced the percentage of H6N2-infected cells by 44% at an MOI of 1 and by 38.6% at an MOI of 5 compared to that of DF-1 cells transfected with vector only (Fig. 3A). A similar restriction of H11N9 by *dIFITM3* was seen with a 30% and 36% reduction at an MOI of 1 and 5, respectively (Fig. 3B). Additionally, to examine whether *dIFITM1* and *dIFITM3* could cooperate in restriction of IAV, we coexpressed both proteins in DF-1 cells. Coexpression of *dIFITM1* and *dIFITM3* resulted in a restriction of both H6N2 and H11N9, similar to that of *dIFITM3* alone (Fig. 3A and B). No reduction in the percentage of infected cells was observed with DF-1 cells transfected with *dIFITM1* or *dIFITM5* compared to that of the vector only despite high expression levels (Fig. 3C). The restriction is obvious in the background of DF-1 cells, which express chicken IFITM3 (32). Transfection efficiency for each construct was approximately 60% (data not shown). Both epitope-tagged and untagged versions of each IFITM variant had similar viral restriction capabilities, with untagged IFITM3 reducing the percentage of infection of H6N2 and H11N9 in DF-1 cells

FIG 3 Duck IFITM3 restricts replication of low-pathogenicity IAV. DF1 cells transiently overexpressing duck IFITMs or empty vector were challenged with H6N2 (A) or H11N9 (B) at an MOI of 1 or 5. Six hours after infection cells were fixed and stained for IAV nucleoprotein, and the percentage of infected cells was determined. The percentage of infected cells is expressed relative to the vector control. Statistical significance compared to the vector control cells was analyzed using an unpaired two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). The maximum percentage of infected cells for H6N2 was 40%, and for H11N9 it was 33%. (C) Expression level of each duck IFITM was determined by Western blotting.

by 33.6% and 44%, respectively, at an MOI of 1 (data not shown). Therefore, the overexpression of dIFITM3 can restrict both H6N2 and H11N9 influenza virus strains in DF-1 cells by 40%, while IFITM1 does not.

Duck IFITMs localize to distinct cellular compartments. To determine which of the duck IFITMs were expressed in the endosomal compartment, we examined the colocalization of each of the duck IFITM proteins with LAMP1, a late endosomal marker. DF-1 cells expressing the different V5-tagged dIFITM proteins were stained with anti-V5 and anti-LAMP1 antibodies to examine colocalization using confocal microscopy (Fig. 4). Similar to mammalian IFITM1, dIFITM1 was present at the cell surface. dIFITM2 colocalizes partially with LAMP1-containing lysosomes but presumably resides in earlier endocytic compartments. dIFITM3 has strong colocalization with endocytic compartments containing LAMP1, consistent with its ability to restrict IAV and previous reports of mammalian and chicken IFITM3 localization.

Interestingly, dIFITM5 also colocalizes partially with LAMP1-containing compartments. Thus, IFITM1 localizes to the cell membrane while IFITM3 is expressed in the endosome, like their mammalian counterparts.

Duck IFITM3 inhibits influenza virus but does not inhibit vesicular stomatitis virus. To further examine the antiviral properties of duck IFITMs, we generated DF-1 clones stably expressing V5-tagged duck IFITMs, challenged them with avian or mammalian viruses, and determined the percentage of infected cells by high-content fluorescence microscopy or flow cytometry. DF1-dIFITM3 cells had 47% fewer H6N2-infected cells, while there was no reduction in the percentage of H6N2-infected DF-1 cells stably overexpressing dIFITM1, dIFITM2, or dIFITM5 (Fig. 5A). Additionally, there was no reduction in the percentage of VSV-GFP-infected cells when any of the dIFITMs was overexpressed (Fig. 5B). Instead, we noted a slight increase in the percentage of infected cells upon overexpression of each dIFITM. To further examine the range of IAV strains restricted by dIFITM3, we challenged DF1-dIFITM3 or vector-only cells with H6N2, H11N9, PR8, and VSV at an MOI of 1. DF1-dIFITM3 cells showed reduced percentages of H6N2-, H11N9-, and PR8-infected cells, 62%, 59%, and 57%, respectively, compared to levels in vector-only cells (Fig. 5C). We confirmed that the decrease in the percentage of H6N2-infected DF1-dIFITM3 cells also corresponded to a decrease in viral titer by plaque assay (Fig. 5D). All stable clones expressed IFITM proteins, although the expression of IFITM2 was low (Fig. 5E). We determined the relative amount of transcription of overexpressed duck IFITMs and endogenous chicken IFITMs using qPCR (Fig. 5F). Duck IFITM1 is expressed 172-fold more than endogenous chicken IFITM1, whereas duck IFITM2 and IFITM3 are expressed 34-fold and 69-fold more than endogenous chicken IFITM2 and IFITM3, respectively. Therefore, the expression level reflects expression induced by an interferon response, and duck IFITM3 restricts the replication of the three influenza strains tested, including avian strains, but does not restrict VSV replication.

The N-terminal domain of dIFITM3 is not essential for antiviral activity or endosomal localization. In domain swapping experiments a chimera of human IFITM3 containing the N-terminal domain of IFITM1 loses association with endosomal compartments and loses its ability to restrict the IAV (35). The N-terminal domain of human IFITM3 is sufficient to cause the localization of human IFITM1 to endocytic compartments but does not result in significant increased antiviral activity (35). To explore whether the N-terminal domain of dIFITM3 functions similarly to the human ortholog, we generated two chimeric proteins to swap the N-terminal domain of dIFITM1 and dIFITM3 and subsequently generated stably expressing DF-1 clones of each chimera (Fig. 6A). DF-1 clones stably expressing dIFITMs or the chimeric proteins were challenged with H6N2 or VSV at an MOI of 1. In contrast to the human chimera, DF-1 cells stably expressing the dIFITM3 chimera containing the N-terminal domain of dIFITM1 (M1M3) retained an ability to restrict IAV and reduced the percentage of H6N2-infected cells by 61% (Fig. 6B). This restriction is comparable to the reduction observed in DF1-dIFITM3 cells. Similar to DF1-dIFITM3 cells, this chimeric protein does not reduce the percentage of VSV-infected cells (Fig. 6C). In fact, there was an observed increase in the percentage of VSV-infected cells that is comparable to the slight increase in DF1-dIFITM3 VSV-infected cells (Fig. 6C). The dIFITM1 chimera containing the N-terminal

FIG 4 Duck IFITMs localize to different cellular compartments. DF1 cells overexpressing dIFITM1, dIFITM2, dIFITM3, or dIFITM5 were fixed, stained, and imaged using confocal microscopy. Panels show staining for Hoechst 33324 (blue), LAMP1 containing endosomes (green), V5-epitope tagged dIFITM (red), and a merged image.

domain of dIFITM3 (M3M1) gained significant antiviral function and reduced the percentage of H6N2-infected cells by 41%, but it does not restrict VSV (Fig. 6B and C). Each protein was expressed at high levels (Fig. 6D). In addition, the M1M3 chimera showed a partial loss of colocalization with LAMP1-containing endosomes (Fig. 6E). This is in contrast to a human M1M3 chimera, which dramatically loses association with endosomes and resembles the staining pattern of IFITM1 (35). Furthermore, the M3M1 chimera results in an increased association with endosomes (Fig. 6E). We quantified the degree of localization of each duck IFITM protein or chimeric protein with LAMP1. Neither dIFITM1 nor dIFITM2 displayed significant colocalization with LAMP1, whereas dIFITM3 and dIFITM5 had significant colocalization with LAMP1 (Fig. 6F). Replacing the N-terminal domain of dIFITM3 with dIFITM1 resulted in decreased association with LAMP1 but not a complete loss of association, analogous to dIFITM1 (Fig. 6F). Exchanging the N-terminal domain of dIFITM1 with that of dIFITM3 increased the association with LAMP1-containing compartments, but this association was not as strong as that for wild-type dIFITM3 (Fig. 6F). Therefore, the N-terminal domain of IFITM3 is not essential for localization to endosomes, yet it can confer partial endosomal localization and antiviral activity on IFITM1.

N-terminal YXX0 endocytic signal sequence of dIFITM3 is not essential for correct cellular localization. The lack of complete mislocalization of the duck IFITM3 chimera containing the N-terminal domain of dIFITM1 away from endosomes led us to further investigate the residues required for dIFITM3 function. Previously, a YXX0 endocytic signal sequence was identified in the N-terminal domain of IFITM3 that is required for correct cellular localization and antiviral function (13, 15). Mutation of the critical tyrosine within this residue is sufficient to achieve the loss of association with endosomes and the loss of antiviral function. To explore whether this conserved sequence was necessary for dIFITM3 function and if other YXX0 motifs were functional within the protein, we mutated four residues of dIFITM3 that appeared to conform to YXX0 endocytosis signal sequences (Fig. 7A) and subsequently generated stably expressing DF1 clones of each mutant. All four mutants colocalized strongly with LAMP1-containing endosomes (Fig. 7B). Duck Y14 is equivalent to human and mouse Y20. dIFITM3-Y14F does not colocalize as strongly as wild-type dIFITM3; however, we did not observe the dramatic relocation of IFITM3 to the cell surface that is seen with mammalian IFITM3 (13). Quantification of the colocalization of dIFITM3 or mutant proteins with LAMP1 shows only that the Y14F mutant has a slight decrease in colocalization compared to

FIG 5 Duck IFITM3 restricts low-pathogenicity IAV but not VSV. DF1 cells stably expressing all dIFITMs were challenged with H6N2 (A) or VSV (B) at an MOI of 1. (C) DF-1 cells expressing dIFITM3 were challenged with H6N2, H11N9, PR8, or VSV at an MOI of 1. The percentage of infected cells was determined using fluorescence microscopy (A) or flow cytometry (B and C). Statistical significance compared to vector control cells was analyzed using an unpaired two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). The maximum percentage of infected cells for H6N2, H11N9, PR8, and rVSV-GFP was 57%, 15%, 12%, and 55%, respectively. (D) Supernatants of DF1 cells stably expressing empty vector or dIFITM3 were collected 12 h postinfection with H6N2, and the viral titer was determined using plaque assay. (E) Level of dIFITM protein expression of each stably expressing DF-1 cell line was determined by Western blotting. (F) Transcript abundance as determined by qPCR of each overexpressed duck IFITM is shown relative to that of its respective endogenous chicken IFITM.

that of wild-type dIFITM3 (Fig. 7C). Of the four point mutants generated from dIFITM3, only Y56F showed a decreased capacity to restrict IAV (Fig. 7D). However, given its endosomal location, we cannot exclude that reduced function is due to lower expression levels than those of the others, although all proteins are overexpressed (Fig. 7E).

IFITM3 dimerization is known to be important for antiviral function (35). One concern was that endogenous chicken IFITM influenced the relative localization of duck IFITMs. However, due to the relatively low abundance of chicken IFITMs in DF-1 cells compared to that of overexpressed duck IFITMs (Fig. 5F), chicken IFITMs likely have a minimal effect on duck IFITM3 antiviral function or cellular localization. In addition, we examined the cellular localization of duck IFITM1, IFITM3, and IFITM3-Y14F in two human cell lines, HeLa and HEK293T (Fig. 8). Duck IFITM1 is expressed pre-

dominantly at the cell surface in both HeLa and HEK293T cells, while duck IFITM3 exists in intracellular punctate structures, likely endosomes. Interestingly, duck IFITM3-Y14F does not accumulate at the cell surface in either HeLa or HEK293T cells. Instead, it exists in intracellular clusters. HEK293 cells are known to express low levels of IFITM3 under basal conditions (37). In contrast, the overexpression of human IFITM3 missing the first N-terminal 21 amino acids (12) or IFITM3-Y20A (13) in HEK293 cells results in the accumulation of the mutant proteins at the cell surface, indicating that low levels of endogenous IFITM3 will not have a major effect on the cellular localization of overexpressed proteins. Taken collectively, the lack of accumulation of dIFITM3-Y14F at the cell surface in both avian and mammalian cells suggests dIFITM3 has critical residues outside the N-terminal domain that are required for correct cellular localization and antiviral function.

FIG 6 N-terminal domain (NTD) of dIFITM3 is not necessary for antiviral activity. (A) Chimeric proteins of dIFITM1 and dIFITM3 were generated. CTD, C-terminal domain. DF1 cells stably overexpressing dIFITM1, dIFITM3, or the chimeric proteins were challenged with H6N2 (B) or VSV (C) at an MOI of 1, and the percentage of infected cells was determined relative to empty vector-transfected cells using fluorescence microscopy (B) or flow cytometry (C). Statistical significance compared to the vector control cells was analyzed using an unpaired two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (D) Expression of each dIFITM or mutant protein was determined using Western blotting. (E) Representative confocal microscopy images of DF1 cells overexpressing 1NTD-3CD225 and 3NTD-1CD225 stained for nuclei (blue), LAMP1 (green), or chimeric protein (red), with a merged image shown. (F) Colocalization of each dIFITM or chimeric protein with LAMP1 was completed using Pearson's correlation coefficient. Bars show mean values from at least 8 analyzed cells.

DISCUSSION

Immune-related IFITMs have the ability to restrict the replication of a broad range of viruses yet show low sequence homology between species. Here, we have functionally characterized the IFITM gene family in the duck, the host and reservoir species for influenza viruses. We show their upregulation in response to influenza virus. We cloned and expressed each IFITM and show IFITM3 localizes to endosomes and has antiviral function against influenza viruses, including avian and mammalian strains. We provide evidence of different signals for endosomal localization of duck IFITM3 than that of mammalian IFITM3. Where mammalian IFITM3 contains an N-terminal endocytic signal sequence that is indispensable for correct cellular localization and antiviral func-

tion (13, 15), domain swapping or mutation of the N-terminal YXX θ motif of duck IFITM3 does not significantly alter endosomal localization or antiviral function. Nonetheless, the N-terminal domain of IFITM3 is sufficient to partially localize IFITM1 to the endosomal compartment, where it then gains antiviral activity against influenza, suggesting the N-terminal domain of dIFITM3 is only partially responsible for endosomal localization.

We used gene synteny and the recently characterized chicken IFITM locus (32) in our annotation of the duck IFITM locus, with genes in the order *IFITM5*, *IFITM2*, *IFITM1*, and *IFITM3* between flanking genes *ATHL1* and *B4GALNT4*. In a phylogenetic tree, mammalian IFITM1, IFITM2, and IFITM3 segregate into one clade, and only avian IFITM3 segregates with this group.

FIG 7 N-terminal YXXθ endocytic signal sequence of dIFITM3 is not necessary for endosomal localization or antiviral activity. (A) Amino acid sequence of duck IFITM3. Regions targeted for mutagenesis are shaded, and the CD225 domain is underlined. (B) Confocal microscopy images of DF-1 cells overexpressing IFITM3, IFITM3-Y14F, IFITM3-Y56F, IFITM3-Y82F, or IFITM3-Y94F. Cells were stained for nuclei (blue), LAMP1 (green), or dIFITM3 protein or mutant protein (red). A merged image is shown. Images are representative of multiple cells analyzed. (C) Colocalization of dIFITM3 or mutant protein with LAMP1 was completed using Pearson's correlation coefficient. Bars show mean values from at least 8 analyzed cells. Statistical significance compared to vector control cells was completed using an unpaired two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (D) DF1 cells stably expressing dIFITM3 or point mutants were challenged with H6N2 at an MOI of 1, and the percentage of infected cells was determined relative to that of vector-only transfected cells by fluorescence microscopy. Statistical significance compared to vector control cells was analyzed using an unpaired two-tailed Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (E) Level of dIFITM protein expression of each stably expressed DF-1 cell line was determined by Western blotting.

IFITM1, IFITM2, and IFITM3 belong to the immune-related clade, whereas IFITM5 and IFITM10 make up the two remaining clades (36). Low sequence identity and gene duplication events of immune-related IFITMs make the assignment of orthologous genes difficult. To reflect the gene synteny with chicken and the function demonstrated here, we have renamed the previously identified duck *IFITM1* gene *IFITM3* (28). There is low sequence identity between mammalian and avian IFITM orthologs, with the exception of IFITM5. The divergence of immune-related IFITMs is thought to be due to selective pressure from viral pathogens specific to each species, whereas IFITM5 possesses a conserved nonimmune role and is under less selective pressure. Alternatively, immune-related IFITMs may have evolved to retain antiviral function while diverging to remain compatible with divergent cellular machinery. However, duck IFITM3 has only 38% identity with human IFITM3, while other components of the en-

docytic machinery are more conserved. For example, adaptor-related protein complex 2, alpha 2 subunit (AP2A2), shows 97% identity between humans and ducks, and a partial sequence for duck VAPA, thought to interact with IFITM3 (38), shows 87% identity between human and ducks. Thus, it seems more likely that immune-related IFITMs have diverged in response to viral pathogens and less to bind cellular machinery components.

Crucially, overexpression of dIFITM3, but not dIFITM1 or dIFITM2, reduces the percentage of IAV-infected DF-1 cells *in vitro*. We show this restriction with IAV strains of both mammalian and avian origins, suggesting dIFITM3 can broadly restrict a wide range of IAV strains. Mammalian IFITMs restrict IAV in a strain-independent manner (5, 6). In addition, dIFITM3 localizes to endosomes, the entry site of IAV. The restriction of influenza viruses by duck IFITM3 is intact despite low amino acid sequence identity. Duck IFITM3 shares only 38% sequence identity with

FIG 8 Cellular localization of duck IFITM1, IFITM3, and IFITM3-Y14F in HeLa and HEK293T cells. HeLa and HEK293T cells were grown on coverslips and transfected with N-terminally V5-tagged duck IFITM1, IFITM3, or Y14F. Cells subsequently were fixed, stained with an anti-V5 antibody (green) and Hoechst 33324 (blue), and imaged using confocal microscopy.

human IFITM3. Notably, dIFITM3 possesses residues that are associated with antiviral activity against influenza virus in mammalian IFITM3, including R87 and Y99, while very few residues in loop 43–52, defined by alanine-scanning mutant 43AS as critical for influenza restriction, were conserved (35).

While IFITM3 has the most antiviral activity against influenza virus, mammalian IFITM1 and IFITM2 can restrict IAV replication to a lesser degree (5). We were unable to demonstrate the antiviral activity of dIFITM2; however, this could be due to low expression levels. Interestingly, dIFITM1 appears to be nonfunctional. dIFITM1 has a longer N-terminal domain with the insertion of a unique repetitive region that appears to have disrupted the gene. It is interesting that while dIFITM1 has the highest upregulation after highly pathogenic IAV infection, we could detect no antiviral activity *in vitro*. However, the protein possesses antiviral activity if partially localized to the endosome by the N-terminal domain of dIFITM3. Interestingly, the chicken homologue of IFITM1 is highly expressed in bursa, ileum, and intestinal tissues (32) and possesses antiviral activity (6). Given that IFITM1 is expressed in intestinal tissues, it perhaps is significant that IFITM1 is nonfunctional in the duck, since this is where low-pathogenicity avian influenza virus replicates (39). Similarly, duck IFITM2 does not appear to be functional against IAV but is functional and expressed in many mucosal tissues in chickens (32). These defects would permit the replication of influenza virus in duck intestine, contributing to its role as the natural host. Meanwhile, dIFITM3 expressed in lung tissue is expected to contribute to the protection

of the duck against highly pathogenic avian influenza virus that replicates in the respiratory tract, as indicated by positive oropharyngeal swabs for VN1203 (28). Ducks sense the presence of IAV through RIG-I and subsequently upregulate ISGs, including dIFITM3, to initiate an innate immune response against highly pathogenic IAV. While chickens lack RIG-I (27), they compensate with the use of MDA5 to detect influenza virus (40, 41) and upregulate ISGs, including functional IFITM1, IFITM2, and IFITM3. Recently, transcriptome analysis of the duck and chicken response to highly pathogenic IAV infection shows a strong upregulation of IFITMs in duck lung tissue after infection but also the absence of significant upregulation of IFITMs in chicken lung tissue (42). This likely contributes to why innate immunity is less protective against highly pathogenic strains in chickens.

Duck IFITM3 resulted in an approximately 50% reduction in IAV-infected DF-1 cells. In contrast, human IFITM3 shows reductions of up to 95% in the percentage of IAV-infected cells (5, 6, 35). However, others show less antiviral activity of IFITM3, with approximately 50% reductions in the percentage of cells infected by IAV (11, 43, 44), VSV (12), or Rift Valley fever virus (45). Two reports have examined the antiviral activity of chicken IFITMs (6, 32). Chicken IFITM1 reduced the infection of murine leukemia virus-GFP pseudotyped with H5 entry proteins by about 70% (6), while the overexpression of chIFITM3 in DF-1 cells reduced WSN infection by 55% compared to that for untransfected controls (32). Perhaps chicken IFITM restricts IAV replication to a lesser extent than mammalian IFITMs; however, it is difficult to compare across different cell types, viral strains, multiplicities of infection, and transfection methods. Whether avian IFITM3 is able to restrict IAV replication as potentially as mammalian IFITM3 or, alternatively, influenza virus strains have evolved mechanisms of partially avoiding restriction by avian IFITMs is not clear. Our data demonstrating duck IFITM3 equally restricts all IAV strains tested, including avian strains, suggests the virus has not evolved to escape restriction.

Interestingly, duck IFITM3 does not reduce the percentage of rVSV-GFP-infected DF-1 cells *in vitro*. This is in contrast to mammalian IFITM3, which can restrict VSV replication (5, 7, 12, 13, 38, 44). IAV requires a pH of approximately 5.0 to 5.7 for membrane fusion, whereas VSV requires a less acidic pH of 6 (46–49). The sites of fusion of viral and host membranes for IAV and VSV correspond to late endosomes/lysosomes and early endosomes, respectively. Additionally, VSV has a two-step mechanism of entry where it fuses with intravesicular bodies before back-fusing with the endosomal membrane to facilitate viral release (50). Neither dIFITM2 nor dIFITM3 could restrict VSV replication. IFITM3 may have a more restricted cellular localization than its mammalian ortholog; therefore, it may be unable to restrict viruses that enter early endocytic compartments. Alternatively, dIFITM3 may not be able to inhibit the fusion of viral membranes with intravesicular bodies or the fusion of intravesicular bodies with the membrane of endosomes. The differences in the entry mechanism between IAV and VSV and sequence differences between mammalian and duck IFITM3 likely account for the differential antiviral activity of dIFITM3 against each virus.

Our chimeric duck proteins show that the N-terminal domain and the tyrosine within the conserved endocytic signal sequence are dispensable for endosomal localization and the function of dIFITM3. This is in contrast to similar chimeric and mutant mammalian IFITM proteins (35). The truncation of the N-termi-

nal domain results in the mislocalization of IFITM3 away from endosomes to the cell periphery (12). In addition, mutation of the tyrosine within the N-terminal YXX θ endocytic signal sequence is sufficient to achieve the loss of association with endosomes and loss of antiviral function (13), while the phosphorylation of Y20 results in mislocalization to the cell membrane (15). Further, initial analysis of a minor allele of IFITM3 (rs12252-C), identified in patients hospitalized with IAV infections, that generates a truncated protein missing the first 21 amino acids (19) showed it was unable to restrict IAV replication *in vitro*. Consistent with our results, a single report of antiviral function in the rs12252-C allele suggests that neither deletion of the N-terminal 21 amino acids of IFITM3 nor mutation of Y20 resulted in a loss of antiviral function (51). The authors of that report suggested that epitope tags influence cellular localization or antiviral function of IFITM3 (51); however, we observed no effect of the addition of an N-terminal V5 tag on the antiviral activity of duck IFITM3 (data not shown). Several explanations may account for the contrasting results from this study, including effects from overexpression or interactions with endogenous proteins. Because our studies were performed with overexpressed IFITM proteins, we cannot rule out all artifacts due to the high level of expression. However, two of our tested IFITM proteins showed no function, demonstrating overexpression did not have a nonspecific antiviral effect. Also, experiments done using unsorted DF-1 cell populations stably expressing dIFITM3, which include cells that express dIFITM3 at low levels (data not shown), still show efficient restriction of IAV replication. Thus, for mammalian IFITM3, endosomal localization is controlled primarily through its N-terminal YXX θ signal sequence, whereas duck IFITM3 appears to require additional signals for internalization.

While the N-terminal domain of IFITM3 is not essential for endosomal localization of dIFITM3, the duck M3M1 chimera mislocalized IFITM1 away from the cell surface partially toward endosomes with a staining pattern that was similar to that of wild-type IFITM3, yet it did not associate with endocytic compartments as strongly as wild-type dIFITM3. This chimera also shows increased antiviral function, suggesting the N-terminal domain is involved in the regulation of cellular localization of duck IFITMs. We also investigated the potential contribution of other putative YXX θ motifs that are present in duck IFITM3 but not conserved in the human or mouse orthologs. DF-1 cells stably expressing the Y56 mutant of dIFITM3 to high levels could not be generated, perhaps because this residue is within a predicted membrane-spanning domain and is important for the structure of dIFITM3. Y82 and Y94 reside within the cytosolic loop region of the CD225 domain. While Y82 does not conform to a canonical YXX θ motif, it closely resembles a typical YXX θ sequence. Major histocompatibility complex class I molecules use a YXXA sequence to regulate internalization from the cell surface (reviewed in reference 52), so we considered that a similar sequence could be used by duck IFITM3. However, mutation of either Y82 or Y94 did not result in a loss of association with endocytic compartments or decreased antiviral function. Our results demonstrate the N-terminal YXX θ sequence of dIFITM3 contributes to endosomal localization, yet other signals outside the N-terminal domain also contribute to proper cellular localization.

Posttranslational modifications contribute to IFITM cellular localization and antiviral function (reviewed in reference 53), including S-palmitoylation (11), tyrosine phosphorylation (15),

ubiquitinylation (14), and methylation (54). Palmitoylation at residues C71, C72, and C105 induces membrane association and clustering (11, 14), and C71 and C72 are conserved in duck IFITM3. Phosphorylation at Y20 results in relocalization of IFITM away from the endosome and also impairs the ubiquitination of IFITM3 (15). This tyrosine is also part of a PPxY recognition site used by the E3 ligase NEDD4 (55), which influences degradation by attaching ubiquitin at four sites (K24, K83, K88, and K104) (14). The recognition site is notably different in birds with PPY instead of PPxY, but ubiquitination sites K83, K88, and K104 are conserved while K24 is absent. Finally, since NEDD4 is inhibited by the ubiquitin modifier ISG15, which is normally induced by infection (56, 57) but is absent from birds (58), we might expect ubiquitin-mediated regulation of IFITM3 to differ. While we have not yet analyzed protein modifications, many key residues are conserved and likely important for localization and function.

Our results demonstrate that IFITMs contribute to the innate immune response of the natural host of IAV, particularly in lung tissues where highly pathogenic avian influenza virus strains replicate. Despite low sequence identity of duck IFITM3 to human IFITM3, residues implicated in influenza virus restriction are conserved, and it retains the ability to restrict influenza virus. While IAV exploits the duck as a natural host and reservoir, this has not led to the evasion of IFITM restriction.

ACKNOWLEDGMENTS

This work was supported by CIHR grant MOP 125865 from the Canadian Institutes of Health Research to K.E.M. This work was funded in part by contract no. HHSN272201400006C from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, and by the American Lebanese Syrian Associated Charities (ALSAC). G.A.D.B. was supported in part by a QEII Graduate Scholarship.

We thank Jerry Aldridge and Patrick Seiler for help with animal work in the biosafety level 3 laboratory of Robert Webster as part of a previous study and Man Rao for help mining the transcriptome for IFITM2. We thank Stephen Ogg and Brittany Fraser for help with the microscopy image analysis.

We declare we have no conflicts of interest and no competing financial interests.

FUNDING INFORMATION

American Lebanese Syrian Associated Charities (ALSAC) provided funding to Robert G. Webster. Gouvernement du Canada | Canadian Institutes of Health Research (CIHR) provided funding to Katharine E. Magor under grant number MOP 125865.

Robert G. Webster received funding from the National Institute of Allergy and Infectious Diseases, NIH, under contract number HHSN272201400006C.

REFERENCES

- Smith S, Weston S, Kellam P, Marsh M. 2014. IFITM proteins-cellular inhibitors of viral entry. *Curr Opin Virol* 4:71–77. <http://dx.doi.org/10.1016/j.coviro.2013.11.004>.
- Bailey CC, Zhong G, Huang I-C, Farzan M. 2014. IFITM-family proteins: the cell's first line of antiviral defense. *Annu Rev Virol* 1:261–283. <http://dx.doi.org/10.1146/annurev-virology-031413-085537>.
- Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell* 38:745–755. [http://dx.doi.org/10.1016/0092-8674\(84\)90270-8](http://dx.doi.org/10.1016/0092-8674(84)90270-8).
- Lewin AR, Reid LE, McMahon M, Stark GR, Kerr IM. 1991. Molecular analysis of a human interferon-inducible gene family. *Eur J Biochem* 199: 417–423. <http://dx.doi.org/10.1111/j.1432-1033.1991.tb16139.x>.

5. Brass AL, Huang I-C, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139:1243–1254. <http://dx.doi.org/10.1016/j.cell.2009.12.017>.
6. Huang I-C, Bailey CC, Weyer JL, Radoshitzky SR, Becker MM, Chiang JJ, Brass AL, Ahmed AA, Chi X, Dong L, Longobardi LE, Boltz D, Kuhn JH, Elledge SJ, Bavari S, Denison MR, Choe H, Farzan M. 2011. Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. *PLoS Pathog* 7:e1001258. <http://dx.doi.org/10.1371/journal.ppat.1001258>.
7. Weidner JM, Jiang D, Pan X-B, Chang J, Block TM, Guo J-T. 2010. Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. *J Virol* 84:12646–12657. <http://dx.doi.org/10.1128/JVI.01328-10>.
8. Wilkins C, Woodward J, Lau DT-Y, Barnes A, Joyce M, McFarlane N, McKeating JA, Tyrrell DL, Gale M. 2013. IFITM1 is a tight junction protein that inhibits hepatitis C virus entry. *Hepatology* 57:461–469. <http://dx.doi.org/10.1002/hep.26066>.
9. Lu J, Pan Q, Rong L, He W, Liu S-L, Liang C. 2011. The IFITM proteins inhibit HIV-1 infection. *J Virol* 85:2126–2137. <http://dx.doi.org/10.1128/JVI.01531-10>.
10. Feeley EM, Sims JS, John SP, Chin CR, Pertel T, Chen L-M, Gaiha GD, Ryan BJ, Donis RO, Elledge SJ, Brass AL. 2011. IFITM3 inhibits influenza A virus infection by preventing cytosolic entry. *PLoS Pathog* 7:e1002337. <http://dx.doi.org/10.1371/journal.ppat.1002337>.
11. Yount JS, Moltedo B, Yang Y-Y, Charron G, Moran TM, López CB, Hang HC. 2010. Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. *Nat Chem Biol* 6:610–614. <http://dx.doi.org/10.1038/nchembio.405>.
12. Jia R, Pan Q, Ding S, Rong L, Liu S-L, Geng Y, Qiao W, Liang C. 2012. The N-terminal region of IFITM3 modulates its antiviral activity by regulating IFITM3 cellular localization. *J Virol* 86:13697–13707. <http://dx.doi.org/10.1128/JVI.01828-12>.
13. Jia R, Xu F, Qian J, Yao Y, Miao C, Zheng YM, Liu SL, Guo F, Geng Y, Qiao W, Liang C. 2014. Identification of an endocytic signal essential for the antiviral action of IFITM3. *Cell Microbiol* 16:1080–1093. <http://dx.doi.org/10.1111/cmi.12262>.
14. Yount JS, Karssemeijer RA, Hang HC. 2012. S-palmitoylation and ubiquitination differentially regulate interferon-induced transmembrane protein 3 (IFITM3)-mediated resistance to influenza virus. *J Biol Chem* 287:19631–19641. <http://dx.doi.org/10.1074/jbc.M112.362095>.
15. Chesarino NM, McMichael TM, Hach JC, Yount JS. 2014. Phosphorylation of the antiviral protein interferon-inducible transmembrane protein 3 (IFITM3) dually regulates its endocytosis and ubiquitination. *J Biol Chem* 289:11986–11992. <http://dx.doi.org/10.1074/jbc.M114.557694>.
16. Li K, Jia R, Li M, Zheng Y, Miao C, Yao Y, Geng Y, Qiao W, Albritton LM, Liang C, Liu S. 2014. A sorting signal suppresses IFITM1 restriction of viral entry. *J Biol Chem* 290:4248–4259.
17. Jia R, Ding S, Pan Q, Liu S-L, Qiao W, Liang C. 2015. The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity. *PLoS One* 10:e0118794. <http://dx.doi.org/10.1371/journal.pone.0118794>.
18. Bailey CC, Huang I-C, Kam C, Farzan M. 2012. Ifitm3 limits the severity of acute influenza in mice. *PLoS Pathog* 8:e1002909. <http://dx.doi.org/10.1371/journal.ppat.1002909>.
19. Everitt AR, Clare S, Pertel T, John SP, Wash RS, Smith SE, Chin CR, Feeley EM, Sims JS, Adams DJ, Wise HM, Kane L, Goulding D, Digard P, Anttila V, Baillie JK, Walsh TS, Hume DA, Palotie A, Xue Y, Colonna V, Tyler-Smith C, Dunning J, Gordon SB, Smyth RL, Openshaw PJ, Dougan G, Brass AL, Kellam P. 2012. IFITM3 restricts the morbidity and mortality associated with influenza. *Nature* 484:519–523. <http://dx.doi.org/10.1038/nature10921>.
20. Zhang Y-H, Zhao Y, Li N, Peng Y-C, Giannoulatos E, Jin R-H, Yan H-P, Wu H, Liu J-H, Liu N, Wang D-Y, Shu Y-L, Ho L-P, Kellam P, McMichael A, Dong T. 2013. Interferon-induced transmembrane protein-3 genetic variant rs12252-C is associated with severe influenza in Chinese individuals. *Nat Commun* 4:1418. <http://dx.doi.org/10.1038/ncomms2433>.
21. Warren CJ, Griffin LM, Little AS, Huang I-C, Farzan M, Pyeon D. 2014. The antiviral restriction factors IFITM1, 2 and 3 do not inhibit infection of human papillomavirus, cytomegalovirus and adenovirus. *PLoS One* 9:e96579. <http://dx.doi.org/10.1371/journal.pone.0096579>.
22. Ding S, Pan Q, Liu SL, Liang C. 2014. HIV-1 mutates to evade IFITM1 restriction. *Virology* 454-455:11–24.
23. Zhao X, Guo F, Liu F, Cuconati A, Chang J, Block TM, Guo J-T. 2014. Interferon induction of IFITM proteins promotes infection by human coronavirus OC43. *Proc Natl Acad Sci U S A* 111:6756–6761. <http://dx.doi.org/10.1073/pnas.1320856111>.
24. Kim J, Negovetich N. 2009. Ducks: the “Trojan horses” of H5N1 influenza. *Influenza Other Respir Viruses* 3:121–128. <http://dx.doi.org/10.1111/j.1750-2659.2009.00084.x>.
25. Webster RG, Bean W, Gorman O, Chambers T, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* 56:152–179.
26. Mandl JN, Ahmed R, Barreiro LB, Daszak P, Epstein JH, Virgin HW, Feinberg MB. 2015. Reservoir host immune responses to emerging zoonotic viruses. *Cell* 160:20–35. <http://dx.doi.org/10.1016/j.cell.2014.12.003>.
27. Barber MRW, Aldridge JR, Webster RG, Magor KE. 2010. Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107:5913–5918. <http://dx.doi.org/10.1073/pnas.1001755107>.
28. Vandervan HA, Petkau K, Ryan-Jean KEE, Aldridge JR, Webster JRRG, Magor KE. 2012. Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51:316–324. <http://dx.doi.org/10.1016/j.molimm.2012.03.034>.
29. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. <http://dx.doi.org/10.1093/bioinformatics/btp033>.
30. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard J-F, Guindon S, Lefort V, Lescot M, Claverie J-M, Gascuel O. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 36:W465–W469. <http://dx.doi.org/10.1093/nar/gkn180>.
31. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682. <http://dx.doi.org/10.1038/nmeth.2019>.
32. Smith SE, Gibson MS, Wash RS, 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. <http://dx.doi.org/10.1128/JVI.01443-13>.
33. Huang Y, Li Y, Burt DW, Chen H, Zhang Y, Qian W, Kim H, Gan S, Zhao Y, Li J, Yi K, Feng H, Zhu P, Li B, Liu Q, Fairley S, Magor KE, Du Z, Hu X, Goodman L, Tafer H, Vignat A, Lee T, Kim K-W, Sheng Z, An Y, Searle S, Herrero J, Groenen MAM, Crooijmans RPMA, Faraut T, Cai Q, Webster RG, Aldridge JR, Warren WC, Bartschat S, Kehr S, Marz M, Stadler PF, Smith J, Kraus RHS, Zhao Y, Ren L, Fei J, Morisson M, Kaiser P, Griffin DK, Rao M, Pitel F, Wang J, Li N. 2013. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45:776–783. <http://dx.doi.org/10.1038/ng.2657>.
34. Hach JC, McMichael T, Chesarino NM, Yount JS. 2013. Palmitoylation on conserved and nonconserved cysteines of murine IFITM1 regulates its stability and anti-influenza A virus activity. *J Virol* 87:9923–9927. <http://dx.doi.org/10.1128/JVI.00621-13>.
35. John SP, Chin CR, Perreira JM, Feeley EM, Aker AM, Savidis G, Smith SE, Elia AEH, Everitt AR, Vora M, Pertel T, Elledge SJ, Kellam P, Brass AL. 2013. The CD225 domain of IFITM3 is required for both IFITM protein association and inhibition of influenza A virus and dengue virus replication. *J Virol* 87:7837–7852. <http://dx.doi.org/10.1128/JVI.00481-13>.
36. Zhang Z, Liu J, Li M, Yang H, Zhang C. 2012. Evolutionary dynamics of the interferon-induced transmembrane gene family in vertebrates. *PLoS One* 7:e49265. <http://dx.doi.org/10.1371/journal.pone.0049265>.
37. Weston S, Czieso S, White IJ, Smith SE, Kellam P, Marsh MA. 2014. Membrane topology model for human interferon inducible transmembrane protein 1. *PLoS One* 9:e104341. <http://dx.doi.org/10.1371/journal.pone.0104341>.
38. Amini-Bavil-Olyae S, Choi YJ, Lee JH, Shi M, Huang I-C, Farzan M, Jung JU. 2013. The antiviral effector IFITM3 disrupts intracellular cholesterol homeostasis to block viral entry. *Cell Host Microbe* 13:452–464. <http://dx.doi.org/10.1016/j.chom.2013.03.006>.
39. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84:268–278. [http://dx.doi.org/10.1016/0042-6822\(78\)90247-7](http://dx.doi.org/10.1016/0042-6822(78)90247-7).

40. Liniger M, Summerfield A, Zimmer G, McCullough KC, Ruggli N. 2012. Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J Virol* 86:705–717. <http://dx.doi.org/10.1128/JVI.00742-11>.
41. Karpala AJ, Stewart C, McKay J, Lowenthal JW, Bean AGD. 2011. Characterization of chicken Mda5 activity: regulation of IFN- β in the absence of RIG-I functionality. *J Immunol* 186:5397–5405. <http://dx.doi.org/10.4049/jimmunol.1003712>.
42. Smith J, Smith N, Yu L, Paton IR, Gutowska MW, Forrest HL, Danner AF, Seiler JP, Digard P, Webster RG, Burt DW. 2015. A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* 16:574. <http://dx.doi.org/10.1186/s12864-015-1778-8>.
43. Li K, Markosyan RM, Zheng Y-M, Golfetto O, Bungart B, Li M, Ding S, He Y, Liang C, Lee JC, Gratton E, Cohen FS, Liu S-L. 2013. IFITM proteins restrict viral membrane hemifusion. *PLoS Pathog* 9:e1003124. <http://dx.doi.org/10.1371/journal.ppat.1003124>.
44. Bailey CC, Kondur HR, Huang I-C, Farzan M. 2013. Interferon-induced transmembrane protein 3 is a type II transmembrane protein. *J Biol Chem* 288:32184–32193. <http://dx.doi.org/10.1074/jbc.M113.514356>.
45. Mudhasani R, Tran JP, Retterer C, Radoshitzky SR, Kota KP, Altamura LA, Smith JM, Packard BZ, Kuhn JH, Costantino J, Garrison AR, Schmaljohn CS, Huang I-C, Farzan M, Bavari S. 2013. IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus. *J Virol* 87:8451–8464. <http://dx.doi.org/10.1128/JVI.03382-12>.
46. Galloway SE, Reed ML, Russell CJ, Steinhauer DA. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS Pathog* 9:e1003151. <http://dx.doi.org/10.1371/journal.ppat.1003151>.
47. Clague M, Schoch C, Zech L, Blumenthal R. 1990. Kinetics of pH-activated membrane fusion of vesicular stomatitis virus with cells: stopped-flow measurements by dequenching of octadecylrhodamine fluorescence. *Biochemistry* 29:1303–1308. <http://dx.doi.org/10.1021/bi00457a028>.
48. Gaudin Y, de Kinkelin P, Benmansour A. 1999. Mutations in the glycoprotein of viral haemorrhagic septicaemia virus that affect virulence for fish and the pH threshold for membrane fusion. *J Gen Virol* 80:1221–1229. <http://dx.doi.org/10.1099/0022-1317-80-5-1221>.
49. Roche S, Gaudin Y. 2004. Evidence that rabies virus forms different kinds of fusion machines with different pH thresholds for fusion. *J Virol* 78:8746–8752. <http://dx.doi.org/10.1128/JVI.78.16.8746-8752.2004>.
50. Le Blanc I, Luyet P-P, Pons V, Ferguson C, Emans N, Petiot A, Mayran N, Demareux N, Fauré J, Sadoul R, Parton RG, Gruenberg J. 2005. Endosome-to-cytosol transport of viral nucleocapsids. *Nat Cell Biol* 7:653–664. <http://dx.doi.org/10.1038/ncb1269>.
51. Williams DEJ, Wu W-L, Grotefend CR, Radic V, Chung C, Chung Y-H, Farzan M, Huang I-C. 2014. IFITM3 polymorphism rs12252-C restricts influenza A viruses. *PLoS One* 9:e110096. <http://dx.doi.org/10.1371/journal.pone.0110096>.
52. Lizée G, Basha G, Jefferies WA. 2005. Tails of wonder: endocytic-sorting motifs key for exogenous antigen presentation. *Trends Immunol* 26:141–149. <http://dx.doi.org/10.1016/j.it.2005.01.005>.
53. Chesarino NM, McMichael TM, Yount JS. 2014. Regulation of the trafficking and antiviral activity of IFITM3 by post-translational modifications. *Future Microbiol* 9:1151–1163. <http://dx.doi.org/10.2217/fmb.14.65>.
54. Shan Z, Han Q, Nie J, Cao X, Chen Z, Yin S, Gao Y, Lin F, Zhou X, Xu K, Fan H, Qian Z, Sun B, Zhong J, Li B, Tsun A. 2013. Negative regulation of interferon-induced transmembrane protein 3 by SET7-mediated lysine monomethylation. *J Biol Chem* 288:35093–35103. <http://dx.doi.org/10.1074/jbc.M113.511949>.
55. Chesarino NM, McMichael TM, Yount JS. 2015. E3 ubiquitin ligase NEDD4 promotes influenza virus infection by decreasing levels of the antiviral protein IFITM3. *PLoS Pathog* 11:e1005095. <http://dx.doi.org/10.1371/journal.ppat.1005095>.
56. Malakhova OA, Zhang DE. 2008. ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. *J Biol Chem* 283:8783–8787. <http://dx.doi.org/10.1074/jbc.C800030200>.
57. Okumura A, Pitha PM, Harty RN. 2008. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc Natl Acad Sci U S A* 105:3974–3979. <http://dx.doi.org/10.1073/pnas.0710629105>.
58. Magor KE, Miranzo Navarro D, Barber MRW, Petkau K, Fleming-Canepa X, Blyth GAD, Blaine AH. 2013. Defense genes missing from the flight division. *Dev Comp Immunol* 41:377–388. <http://dx.doi.org/10.1016/j.dci.2013.04.010>.