

Article

Influenza A Virus NS1 Inhibits RIPLET Activation of Duck RIG-I Signaling

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Abstract

Retinoic acid-inducible gene I (RIG-I) is a crucial pattern recognition receptor for detecting viral RNA and initiating an immune response against influenza A viruses (IAVs). The activation of RIG-I in mammalian cells requires ubiquitination by two E3 ubiquitin ligases: TRIM25 and RIPLET. Using dual luciferase assays, we demonstrate that duck RIPLET enhances the activation of RIG-I, driving the IFN- β promoter activity in chicken DF-1 fibroblasts. qPCR analyses show that the co-expression of duck RIG-I and RIPLET significantly upregulates key immune genes and reduces viral RNA transcripts in DF-1 cells challenged with low pathogenic avian influenza (LPAI) H6N2. Co-immunoprecipitation and confocal microscopy studies suggest the interaction and confirm the colocalization of duck RIG-I and RIPLET in the cytoplasm. Further, we show that the non-structural protein 1 (NS1) of IAV, known for its role in immune evasion, suppression, and pathogenicity, from five different strains of IAV (PR8, BC500, CA431, D4AT, and VN1203) can all inhibit duck RIPLET activation of RIG-I, with NS1 from avian strains showing the greatest decrease in IFN- β promoter activity in chicken DF-1 cells. Overall, our research provides valuable insight into the E3 ubiquitin ligases required for RIG-I activation and susceptibility of this pathway to IAV interference across species.

Keywords: duck; RIG-I; RIPLET; influenza A virus; non-structural protein 1

1. Introduction

Ducks are the natural reservoirs for low-pathogenicity avian influenza (LPAI) viruses [1] and show a high degree of resistance to highly pathogenic avian influenza (HPAI) viruses [2–4]. The resistance to HPAI could be partially attributed to the efficient function of their RIG-I-mediated immune responses [5]. In contrast, chickens lack the RIG-I receptor [5]. The inactivation of RIG-I in Galliformes likely occurred approximately 45–65 million years ago (MYA), leading to a complete loss of RIG-I in most species within this group [6]. This loss was consistently accompanied by the disruption of RING finger protein, leading to RIG-I Activation (RIPLET), indicating an intricate relationship between RIG-I and RIPLET in avian species [6]. The deficiency of RIG-I and RIPLET may impair the ability of chickens to detect viral RNA and mount an effective immune response against AIV, making them more susceptible to infection [5,7].

RIG-I is activated by K63-linked polyubiquitination. Initially, TRIM25 was identified as the E3 ubiquitin ligase responsible for K63-linked polyubiquitination and the activation



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of human RIG-I caspase and recruitment domains (CARDs) [8]. Later, ubiquitin ligases RIPLET (also known as REUL or RNF135), Mex3c and TRIM4 were also shown to mediate K63-linked polyubiquitination of human RIG-I [9–11]. However, knockouts of the Mex3c and TRIM4 in HEK293T and MEF cells did not inhibit RIG-I activation, suggesting these E3 ubiquitin ligases were less important for RIG-I activation [12]. Similarly, knockout of TRIM25 did not impair RIG-I activation of innate signaling in human and murine cells [13,14]. Thus, RIPLET appears to be the obligatory E3 ubiquitin ligase for RIG-I activation, not TRIM25 [13,14]. Despite this, multiple studies have shown proteins or RNAs that target TRIM25 and dampen antiviral functions in the context of human RIG-I [15–26]. Additionally, RIPLET can polyubiquitinate LGP2 (laboratory of genetics and physiology 2), a member of the RIG-I-like receptor family, at later stages of virus infection that allows the fine-tuning of RIG-I-induced excessive type I IFN expression [27].

The overexpression of duck RIG-I in chicken DF-1 cells provides a valuable model to study the cross-species functionality and the potential enhancement of antiviral defenses in chickens, which are naturally deficient in RIG-I. Previous studies have successfully knocked-in and expressed duck RIG-I in DF1 cells, demonstrating that duck RIG-I can recognize and respond to poly(I:C) stimulation similarly to its endogenous expression in ducks [28]. In DF-1 cells that are naturally unable to respond to short 5'ppp dsRNA, the overexpression of duck RIG-I only enabled these cells to respond and initiate a cascade of reactions that upregulated type I interferons and interferon-stimulated genes (ISGs) downstream of RIG-I, such as myxovirus resistance 1 (*MX1*), interferon-induced protein with tetratricopeptide repeats 5 (*IFIT5*), 2'-5'-oligoadenylate synthetase-like protein (*OASL*), protein kinase R (*PKR*), and interferon-stimulated gene 12 (*ISG12*) [5,7]. The overexpression of duck RIG-I driven by a strong CMV promoter in DF-1 cells established an antiviral state and reduced viral titers when infected with LPAI (H5N2) or HPAI (H5N1) [7]. In contrast, a more recent study using chicken embryonic fibroblasts (CEFs) and transgenic chickens expressing duck RIG-I under its native promoter reported no significant reduction in LPAI (H9N2) replication [29]. This is likely attributable to a stronger expression of RIG-I driven by the CMV promoter [7] compared with the duck RIG-I promoter in chicken cells [29]. The authors also noted that differences in influenza virus subtypes and the cell types used may have contributed to the contrasting findings [29]. In vivo experiments using transgenic chickens expressing duck RIG-I alone or together with duck RIPLET further revealed unexpected outcomes; instead of providing protection, these proteins exacerbated disease, caused persistent weight loss, heightened inflammatory responses, and increased mortality following AIV infection [29]. The authors also suggested that the evolutionary loss of RIG-I and RIPLET in chickens may have been advantageous, because it reduced the risk of excessive inflammation during infection [29].

The non-structural protein 1 (NS1) of IAV plays a significant role in evading the host immune response by inhibiting RIG-I-mediated signaling in a species-specific manner. NS1 has also been shown to interact with TRIM25, effectively blocking its function in the activation of RIG-I CARD-initiated signaling [30]. In human cells, NS1 from human-, avian-, swine- and mouse-adapted strains could all bind to TRIM25; however, only the NS1 protein from the human strain was able to bind human RIPLET and inhibit RIPLET-dependent RIG-I-mediated signaling [31]. In mice, NS1 from these tested strains could only bind and inhibit the IFN- β promoter activity through binding to mouse RIPLET and not TRIM25 [31].

NS1 also interferes with the host mRNA maturation through its interaction with CPSF30, effectively inhibiting its function and thereby preventing the polyadenylation of host cell pre-mRNAs [32], leading to an overall non-specific decrease in host protein synthesis [32–36]. Mutating NS1 at the G184 residue disrupts the interaction with CPSF30 but does not impact the ability of PR8 NS1 to bind to TRIM25 or inhibit interferon induction

in HEK293T cells [37,38]. Interestingly, the avian NS1-G184R mutants effectively downregulated the IFN- β promoter activation and ubiquitination of human GST-2CARD in HEK293 cells but did not affect the function of duck TRIM25 and duck GST-2CARD expressed in chicken DF-1 cells [37]. NS1 from PR8 does not inhibit TRIM25 ubiquitination of h2CARD, yet the virus efficiently decreases IFN- β release from HEK cells [37].

We previously thought that TRIM25 was the main E3 ubiquitin ligase involved in the activation of duck RIG-I [39,40], as we were unable to recover a RING domain in duck RIPLET in 5'RACE experiments, likely due to its high GC content [39,40]. A full-length duck RIPLET transcript containing the RING domain was later predicted and used in the transgenic chicken study [29]. We and others have demonstrated that duck RIG-I overexpressed in chicken DF-1 cells can function [5,7,28] and these cells do not have RIPLET, suggesting some redundancy in E3 ubiquitin ligases used. We and others have demonstrated that the suppression of IFN signaling is due to NS1 inhibiting ubiquitination by RIPLET or TRIM25 [30,31,37], although TRIM25 studies were performed with 2CARD. More recently, data suggest that RIPLET is the essential E3 ubiquitin ligase for RIG-I activation [13,14,41,42] and TRIM25 has RIG-I independent functions [41,43,44].

In this study, we confirm the full-length duck RIPLET transcript using transcriptomic data [45] and PCR amplification from duck lung tissue infected with IAV from a previously described study [5]. To examine the contribution of RIPLET to duck RIG-I signaling in avian cells, we measured the downstream effects on IFN- β promoter activity, ISG induction, and antiviral responses. We also show that NS1 from different strains of IAV can inhibit RIPLET-activated duck RIG-I signaling.

2. Materials and Methods

2.1. Cell Culture and Transfections

DF-1 cells, a chicken embryonic fibroblast cell line derived from an East Lansing line (EV-0) embryo [46], were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded overnight in either 24-well plates at a concentration of 3×10^5 per well in 0.5 mL of media or in 6-well plates at 6×10^5 cells per well in 2 mL of media. The following day (24 h), cells were transfected with the indicated DNA constructs using Lipofectamine 2000™ reagent (Invitrogen, Waltham, MA, USA) at a DNA to Lipofectamine ratio of 1:2.5. Six hours post-transfection, cells were additionally transfected with either 19 bp 5'ppp-dsRNA, a dephosphorylated 19 bp dsRNA (InvivoGen, San Diego, CA, USA), or with no ligand.

2.2. Plasmids and Constructs

All constructs in this study utilized the pcDNA3.1 (Hygro+) plasmid (Invitrogen, Waltham, MA, USA) as the backbone, and Phusion® High-Fidelity PCR Master Mix (NEB) for PCR. Duck RIPLET was amplified from cDNA (Table 1) derived from RNA extracted from lung tissues infected with H5N1 A/Vietnam/1203/04 (VN1203) following a previously described viral challenge experiment [5]. The duck RIPLET sequence was deposited in GenBank with accession number PV972220. The PCR product was initially cloned into pCR2.1-TOPO (Invitrogen, Waltham, MA, USA), sequenced, and subsequently cloned into the pcDNA3.1/Hygro(+) expression vector using *BmtI* and *HindIII* restriction sites, with a C-terminal HA-tag included. Duck RIG-I-3xFlag and TRIM25-V5 constructs were created as described by Miranzo-Navarro & Magor [47], while Flag-NS1 expression constructs were created by Evseev et al. [37]. WT-NS1 and G184R-NS1 constructs used in this study are from the following viruses: A/duck/British Columbia/500/2005 [H5N2], A/duck/D4AT/71.1/2004 [H5N1], A/Vietnam/1203/2004 [H5N1], A/chicken/California/431/2000 (H6N2) and A/Puerto Rico/8/1934 [H1N1].

Plasmids were transformed into *Escherichia coli* DH5 α , with individual clones selected for plasmid isolation using the Presto™ Mini Plasmid Kit (Geneaid, New Taipei City, Taiwan). High-purity plasmids for transfection were prepared using the Presto™ Midi Plasmid Kit (Geneaid, New Taipei City, Taiwan).

Table 1. List of primers used.

Fragment	Primer	Primer Sequence (5'→3')
dRIPLET	Forward	ATGGCTGTCCCAGCGGAG
dRIPLET	Reverse	GCTAAACACTGAAGCTGGCATAGAAGC
Bmt1-dRIPLET	Forward	ATTAGCTAGCATGGCTGTCCCAGCG
HindIII-stop codon-HA-dRIPLET	Reverse	TAATAAGCTTTTAAGCGTAATCTGGAACATCGTATGGGTACTTCTGTTTATGTGATTATAGTT
Chicken ISG12-2	Primer 1	TGCTTCATCGATCATGTC TGG
Chicken ISG12-2	Primer 2	GAGATCCCCTGCTGGTAAAG
Chicken ISG12-2	Probe	/56-FAM/TG TTG AGC C/Zen/T TTG GCA CCC ATT TC/3IABkFQ/

2.3. Dual Luciferase Reporter Assays

The chicken IFN- β promoter luciferase reporter plasmid (pGL3-chIFN β), derived from the chicken IFN2 gene, was utilized as described by Barber et al. [5]. DF-1 cells were seeded in 24-well plates at 3×10^5 cells per well and transfected 24 h later with 150 ng of the IFN- β reporter plasmid, 10 ng of the synthetic *Renilla* luciferase internal control construct phRTK, and 150 ng (unless specified otherwise) of each RIG-I or 2CARD, and RIPLET or TRIM25 constructs in the pcDNA3.1/Hygro(+) backbone vector per well. Total transfected DNA was kept consistent by the addition of empty vector DNA. Six hours post-transfection, cells were transfected with 250 ng of 19 bp 5'ppp-dsRNA, or 250 ng of dephosphorylated 19 bp dsRNA (InvivoGen, San Diego, CA, USA), or with no ligand. After 20 h, cells were lysed with 100 μ L of passive lysis buffer per well for 30 min at room temperature. The cell lysate (20 μ L) was transferred to a tube containing 100 μ L of luciferase assay reagent II, and firefly luciferase activity was measured using a GloMax 20/20 Luminometer (Promega, Madison, WI, USA). Subsequently, 100 μ L of Stop & Glo® reagent was added, and *Renilla* luciferase activity was measured. All treatments were performed in triplicate across three independent experiments.

2.4. Immunoprecipitation and Immunoblotting

DF-1 cells in 6-well plates were transfected with 1 μ g of each DNA construct, followed by an additional transfection with 1 μ g of 19 bp 5'ppp-dsRNA, dephosphorylated 19 bp dsRNA (InvivoGen, San Diego, CA, USA), or no ligand 6 h later. After 20 h, cells were lysed in 1 mL of lysis buffer (50 mM TRIS pH 7.2, 150 mM NaCl, 1% [vol/vol] Triton X-100, protease inhibitor cocktail [Roche]), sonicated for 10 s, and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was divided into three parts: 150 μ L for whole cell lysate (WCL) samples and two 400 μ L aliquots for Flag and HA immunoprecipitations (IP). IP samples were incubated for 1.5 h with 1 μ g of anti-Flag-M2 monoclonal antibodies (F3165; Sigma-Aldrich, St. Louis, MO, USA) for Flag IP or anti-HA rabbit monoclonal antibodies for HA IP (A191-102, [BLRE02G], Bethyl Laboratories, Montgomery, TX, USA), followed by mixing with 30 μ L of Pierce™ Protein G Magnetic Beads (Thermo Scientific, Waltham, MA, USA) for 10 min and washing three times with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). Beads were boiled in 2xLaemmli buffer for 5 min. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon®-P membrane, MilliporeSigma, Burlington, MA, USA) with a pore size of 0.45 μ m. Each target protein was run on a separate membrane. Proteins were detected using anti-HA HRP (1:1000) (901519, Direct-Blot™ HRP anti-HA.11 Epitope Tag, BioLegend, San Diego, CA, USA) and anti-Flag rabbit (1:5000) (ab205606, Abcam, Cambridge, UK) antibodies, visualized

by chemiluminescence with Pierce[®] ECL Western blotting substrate (Thermo Scientific). Images were acquired using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). For chemiluminescence, the system automatically determined the optimal exposure for each blot. For the colorimetric channel, the rapid auto-exposure function was applied.

2.5. Virus Infections in Cell Culture

The titer of the viral stock was determined by plaque assay on MDCK cells. DF-1 cells were seeded in 24-well plates and transfected with 0.5 µg of each of RIG-I, RIPLET or ΔRING-RIPLET construct, adjusted with pcDNA to a total of 1 µg per well. After 24 h, cells were infected with low pathogenic A/Ck/California/431/2000 (H6N2) “CA431” or H6N2 at a MOI of 1 in the presence of 0.1 µg/mL tosylamido-2-phenyl ethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA). Cells were incubated for 1 h with H6N2 (infected) or with no virus (mock infected). Fifteen hours later, cells were washed with warm 1xPBS, trypsinized with 150 µL Trypsin-EDTA (0.25%), and 300 µL serum-free EDTA, transferred to tubes, and centrifuged for 5 min at 400 rpm at 4 °C. The cell pellets were subjected to RNA extraction using the PuroSPIN[™] Total RNA Purification Kit (#NK051-250).

2.6. RT-qPCR Analysis

RNA was treated with DNase, and first-strand cDNA was synthesized using random hexamer primers (Invitrogen, Waltham, MA, USA) and the SuperScript III reverse transcriptase kit (Invitrogen, Waltham, MA, USA). Gene-specific primers and probe sets (Table 1) were as described by Barber et al. [7], except for chicken ISG12-2 primers, which were designed using the IDT RealTime qPCR tool. Primers were obtained from Integrated DNA Technologies and validated for linear amplification and relative amplification efficiency matched to the GAPDH endogenous control. qRT-PCR assays were performed using Fast Start TaqMan Probe Master Mix (Roche) on the QuantStudio[™] 3 System (Thermo Fisher Scientific, Waltham, MA, USA). Experimental samples were assayed in triplicate, with target gene expression normalized to GAPDH. Quantification was performed using the ΔΔCT method, analyzed with QuantStudio Design and Analysis Software v1.5.1 (Applied Biosystems, Waltham, MA, USA).

2.7. Confocal Microscopy and Colocalization Analysis

DF-1 cells were seeded into 4-well chamber slides (C6807, Nunc[®] Lab-Tek[®] II Chamber Slide[™] system, MilliporeSigma, Burlington, MA, USA) or 24-well plates and 12 mm, #1½ circular cover glass (Electron Microscopy Sciences, 72230-01) at 3×10^5 cells per well and transfected with 0.5 µg of each DNA construct to a total of 1 µg per well. Six hours later, cells were transfected with 500 ng of dsRNAs or no ligand. After 24 h, cells were fixed in 4% paraformaldehyde (PFA) for 10 min, permeabilized with PBS + 0.25% Triton X-100 for 10 min and blocked in 4% BSA for 1 h. Subcellular distributions of proteins were determined by staining with mouse anti-Flag conjugated to Alexa 488 (Abcam, Cambridge, UK) and rabbit anti-HA monoclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA), followed by a secondary goat anti-rabbit antibody conjugated to Alexa Fluor 561 (Thermo Scientific, Waltham, MA, USA). Nuclei were stained with Hoechst 33342 (Life Technologies). Chicken mitochondria were stained using 100nM MitoTracker[™] Red CMXRos (M7512, Invitrogen, Waltham, MA, USA). Cells expressing RIPLET-HA and Flag-NS1 were fixed in 2% PFA and subcellular distributions of proteins were determined by staining with mouse anti-Flag conjugated to Alexa 647 (Abcam, Cambridge, UK) and rabbit anti-HA monoclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA), followed by a secondary goat anti-rabbit antibody conjugated to Alexa Fluor 488.

Images were captured on an Olympus FLUOVIEW FV3000 confocal laser scanning microscope at a resolution of 512 by 512 pixels with Z-stacks ranging from 4 to 10 μm . Z-stacks were deconvoluted using the constrained iterative function (10 iterations) in cellSens V4.3. For total IntDen analysis, deconvoluted Z-stacks were collapsed into a single image using the average intensity projection method in FIJI (ImageJ). Regions of interest (ROIs) for nuclei were selected by thresholding the Hoechst channel at 500, while the Flag-NS1 (647) channel was thresholded at 1000. Colocalization analysis was performed using the BIOP version of JACoP plugin across the Z-stacks (retrieved from <https://github.com/BIOP/ijp-jacop-b> [accessed on 5 May 2024]) in FIJI (ImageJ) [48].

3. Results

3.1. Duck RIPLET Activation of RIG-I Signaling and Downstream Chicken IFN- β Promoter Activity

To determine the effect of duck RIPLET on the activation of the chicken IFN- β promoter, we conducted dual luciferase assay experiments in DF-1 fibroblasts. Promoter activation was measured upon expression of various constructs including RIG-I, TRIM25, RIPLET, or RIG-I CARDs (2CARD) followed by stimulation with 19 bp dsRNA, 5'ppp 19 bp dsRNA, or no ligand. Duck RIPLET significantly increased RIG-I activation of chicken IFN- β promoter activity in the presence of 5'ppp 19 bp dsRNA (Figure 1A). In contrast, Δ RING-RIPLET, which lacks the RING domain, failed to enhance signaling, underscoring the crucial role of the RING domain in IFN- β production (Figure 1A).

We compared molar equivalent amounts of constitutively active N-terminal CARD domains from duck RIG-I (2CARD) and full-length duck RIG-I with RIPLET for ability to stimulate chicken IFN- β promoter activity (Figure 1B). RIG-I-expressing cells matched the levels seen with 2CARD when in the presence of RIPLET in a dose-dependent manner, with higher amounts of RIPLET allowing for greater RIG-I dependent stimulation by 5'ppp 19 bp dsRNA (Figure 1B). Our avian system revealed that the co-expression of duck RIPLET and RIG-I is sufficient to significantly upregulate IFN- β promoter activity, even in the absence of any ligands (Figure 1C). However, RIG-I still demonstrated significant responsiveness to both dsRNA and 5'ppp dsRNA ligands (Figure 1C).

While duck RIPLET is the stronger activator of duck RIG-I-dependent IFN- β production, the co-transfection of duck TRIM25, RIPLET and RIG-I resulted in the highest activation of the chicken IFN- β promoter, suggesting that TRIM25 and RIPLET co-operate to enhance downstream signaling (Figure 1D). Further examination of the effect of duck TRIM25 on duck 2CARD domains revealed both positive and negative impacts on the IFN- β response. Duck TRIM25 and RIPLET each activated signaling when co-expressed with duck 2CARD at low concentrations (Figure 1E). However, at higher concentrations of duck 2CARD, the IFN- β response was downregulated by duck TRIM25 (Figure 1F). However, 2CARD at this high concentration maximally activated downstream signaling, as RIPLET did not increase promoter activation.

3.2. Enhancement of Antiviral Immunity by Co-Expression of Duck RIG-I and RIPLET in DF-1 Cells Challenged with LPAI (H6N2)

To assess the potential enhancement of antiviral immunity by RIG-I and RIPLET during an influenza infection, we examined the expression of IFN- β and interferon-stimulated genes by qPCR. After transfecting DF-1 cells with duck RIG-I alone, RIG-I and RIPLET or RIG-I and Δ RING-RIPLET, we challenged them with A/Ck/CA431/2000 (H6N2). We measured the relative amounts of mRNA transcripts of IFN- β , MX1, OASL, PKR, ISG-12.2 and IFIT5, and the M1 gene of IAV. In all three independent experimental trials, the co-expression of RIG-I with either RIPLET or Δ RING-RIPLET significantly enhanced the

expression of genes downstream of the RIG-I signaling pathway, leading to reduced relative viral Matrix transcripts (Figure 2). We note that these ISGs were upregulated in mock infected cells co-expressing duck RIG-I with RIPILET or Δ RING-RIPILET, which is sufficient to stimulate antiviral pathways. Upon infection, we noticed significant decreases in IFN- β transcripts compared with mock infections, suggesting that the virus decreases its expression. Although the upregulation values varied among experiments, the trend remained consistent, with the highest values observed in RIG-I and RIPILET co-expression. The overexpression of RIG-I alone, as well as RIG-I and Δ RING-RIPILET co-expression, also showed a significant upregulation of ISGs (Figure 2).

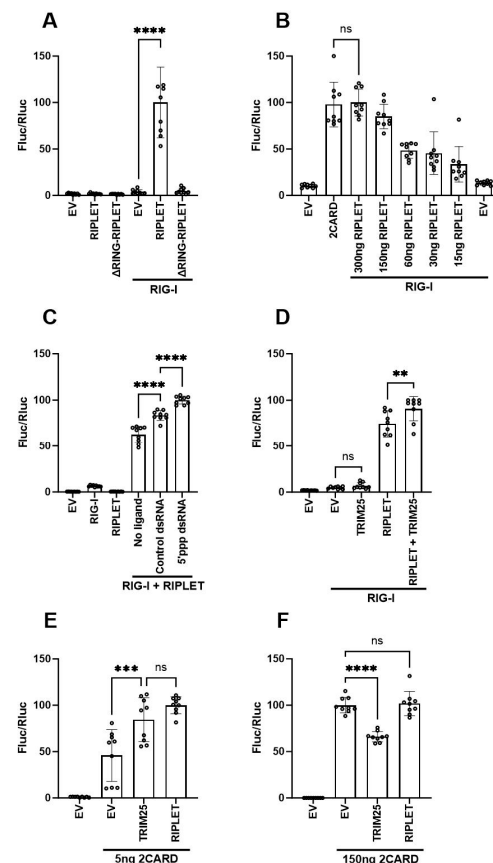


Figure 1. RIPILET enhances relative chicken IFN- β promoter activity when co-transfected with RIG-I in chicken DF-1 cells. EV—empty vector or pcDNA3.1 (Hygro+) plasmid; Fluc/Rluc—relative ratio of Firefly luciferase under control of the chicken IFN- β promoter to *Renilla* luciferase. (A) RIPILET and RIG-I elicit significantly greater IFN- β promoter activity compared with RIG-I alone, and this effect requires the catalytic RING domain of RIPILET. (B) RIPILET enhances RIG-I activation of the IFN- β promoter in a dose-dependent manner, equivalent to activation by the constitutively active RIG-I N-terminal end (2CARD). Cells were transfected with molar equivalents of 2CARD (from duck RIG-I) and full-length duck RIG-I at low concentrations (20 ng/well and 27 ng/well, respectively) and increasing concentrations of RIPILET. (C) Co-expression of RIG-I and RIPILET significantly increases chicken IFN- β promoter activity even in the absence of ligands, but activation is increased with either dsRNA or 5'ppp-dsRNA ligands. (D) Co-expression of TRIM25 and RIPILET (150 ng of each construct) enhances RIG-I-stimulated IFN- β promoter activity more than RIPILET alone (150 ng). (E) Either TRIM25 or RIPILET stimulate IFN- β promoter activity when co-transfected with 5 ng of constitutively active RIG-I N-terminal CARD domains (2CARD). (F) TRIM25 has a negative effect on IFN- β promoter activity at a higher concentration of 2CARD (150 ng), whereas RIPILET has no effect. GraphPad prism 10 software was used for Tukey's multiple comparison under one-way analysis of variance (ANOVA) (** = $p < 0.001$, *** = $p < 0.0001$, **** = $p < 0.00001$, ns: not significant.).

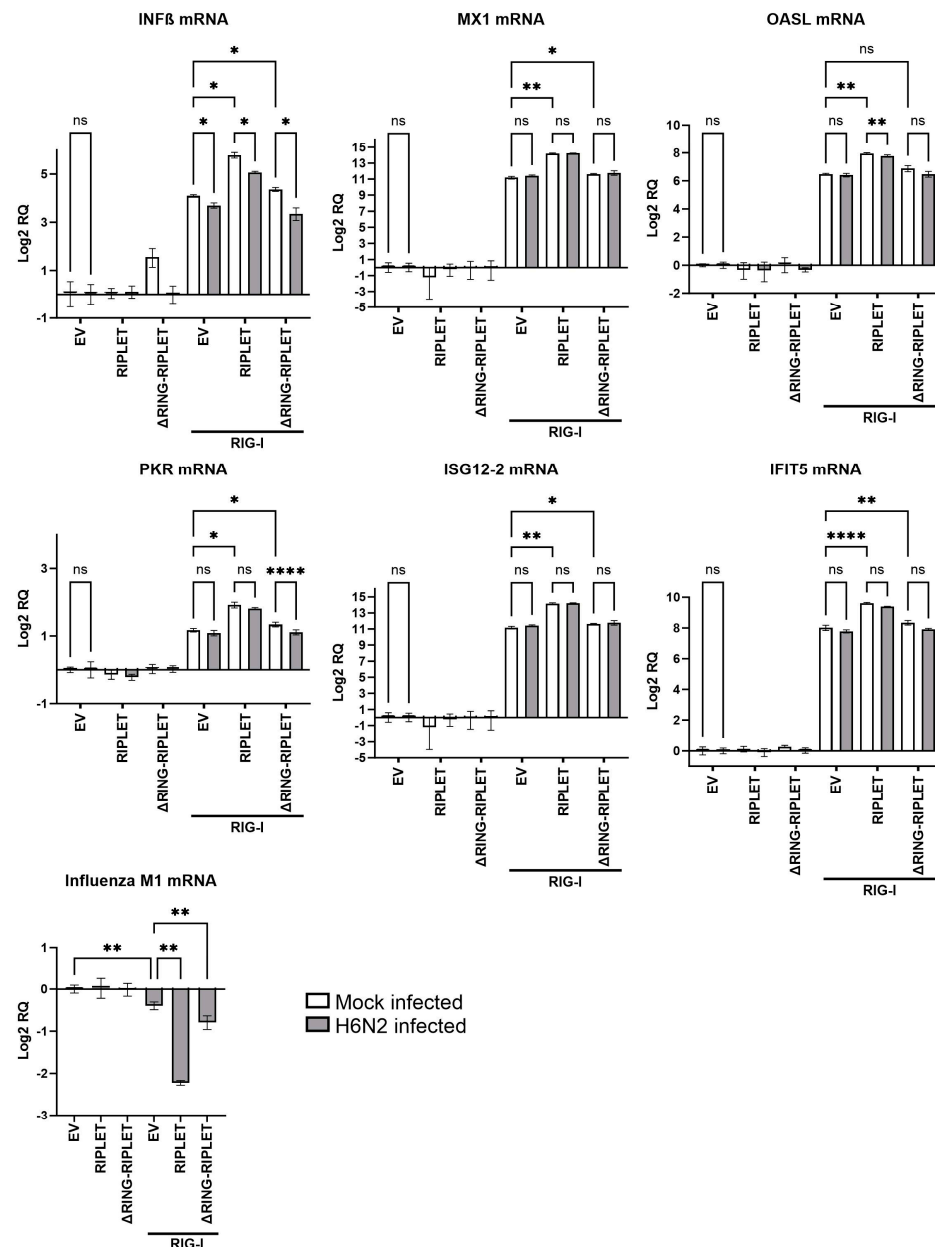


Figure 2. Upregulation of innate immune genes in chicken DF-1 cells expressing duck RIG-I and RIPLET, and infection with low pathogenic AIV. Transcripts of IFN- β , MX1, OASL, PKR, ISG12, IFIT5 and influenza M1 are shown; error bars show RQMin/Max at a 95% confidence level. Transfection was conducted using 0.5 μ g of each construct, adjusted with empty pcDNA3.1/Hygro(+) vector (EV) to a total of 1 μ g per well. Twenty-four hours post-transfection, cells were infected with low pathogenic A/Ck/California/431/2000 (H6N2) “CA431” at a MOI of 1. RNA extraction was performed 15 h post-infection, followed by cDNA synthesis. Target gene expression was normalized to the constitutively expressed endogenous control gene GAPDH. One experiment of three is shown. The linear scale was changed to a Log2 scale on GraphPad prism 10 and Tukey’s multiple comparison under one-way analysis of variance (ANOVA) was performed (* = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.00001$, ns: not significant.).

3.3. Colocalization Analysis of Duck RIG-I, RIPLET, and Mitochondria with and Without Ligand Stimulation

To determine if duck RIG-I colocalizes with duck RIPLET and/or mitochondria, and if these distributions change in response to a 5' ppp ligand, we set up confocal microscopy experiments. In these experiments, we overexpressed tagged RIG-I and RIPLET and used

antibodies with fluorophores and MitoTracker stain to visualize potential colocalization. The distribution of RIG-I resembles the distribution pattern of RIPLET, both having a diffuse distribution mostly in the cytoplasm (Figure 3A). However, we did not observe a high degree of colocalization between RIG-I and mitochondria (Figure 3B). Colocalization of RIG-I and RIPLET was confirmed by high Pearson's and Manders' colocalization coefficients (Figure 3C), while correlation of signals for RIG-I and mitochondria was low. The subcellular distributions did not change in the presence of 5'ppp dsRNA. Mitochondria displayed a distinct pattern in the cells, whereas RIG-I was more dispersed. The addition of 5'ppp dsRNA did not alter this RIG-I distribution pattern, and it did not colocalize with mitochondria.

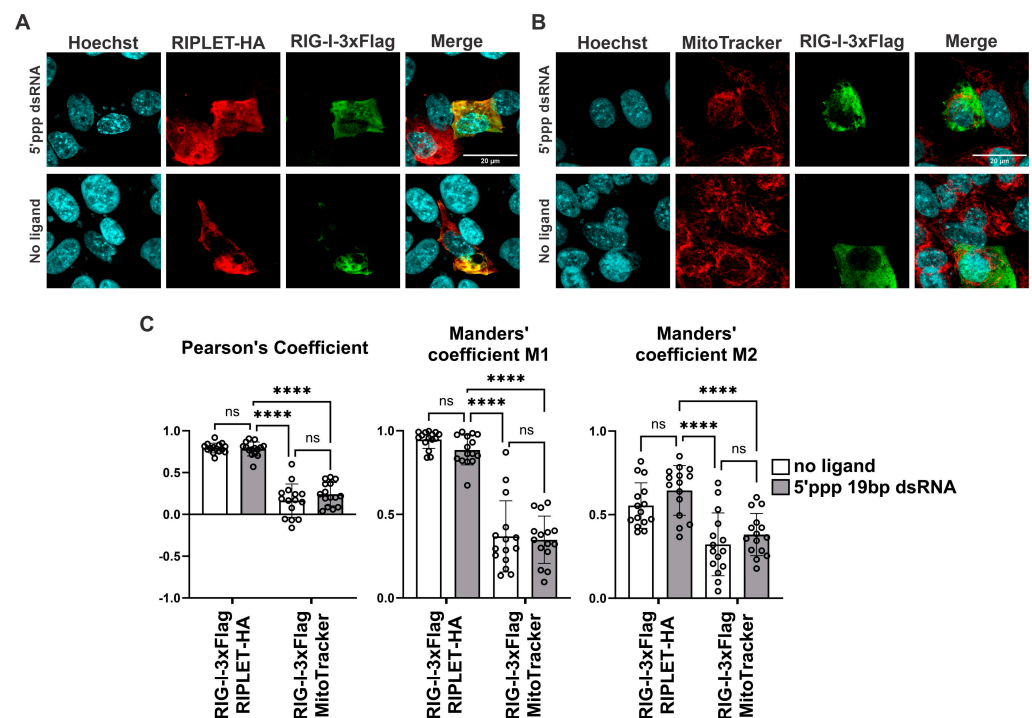


Figure 3. Subcellular localizations of duck RIG-I, RIPLET and mitochondria. **(A)** Representative image showing subcellular distribution of duck RIG-I and RIPLET. **(B)** Representative image of duck RIG-I and mitochondria stained with MitoTracker. **(C)** Graphs showing Pearson's correlation coefficient and Manders' colocalization coefficients (M1 and M2). Images were analyzed with Fiji (ImageJ). Each individual channel is represented by separate images. The nucleus is shown in cyan, RIG-I in green, RIPLET and MitoTracker both in red; $n = 15$ cells per group, statistical significance was assessed using Tukey's multiple comparison under one-way analysis of variance (ANOVA) (**** = $p < 0.00001$, ns: not significant.).

3.4. Co-Immunoprecipitation of Duck RIG-I and RIPLET with and Without Ligand

To test whether duck RIG-I and RIPLET co-immunoprecipitate and whether a specific ligand could increase the number of proteins pulled down, we set up an experiment similar to the one in the luciferase assays (Figure 1C). Our results indicate that duck RIG-I and RIPLET co-immunoprecipitated with either protein serving as bait (Figure 4). Additional transfection with either dsRNA or 5'ppp dsRNA did not increase the amount of interacting protein pulled down.

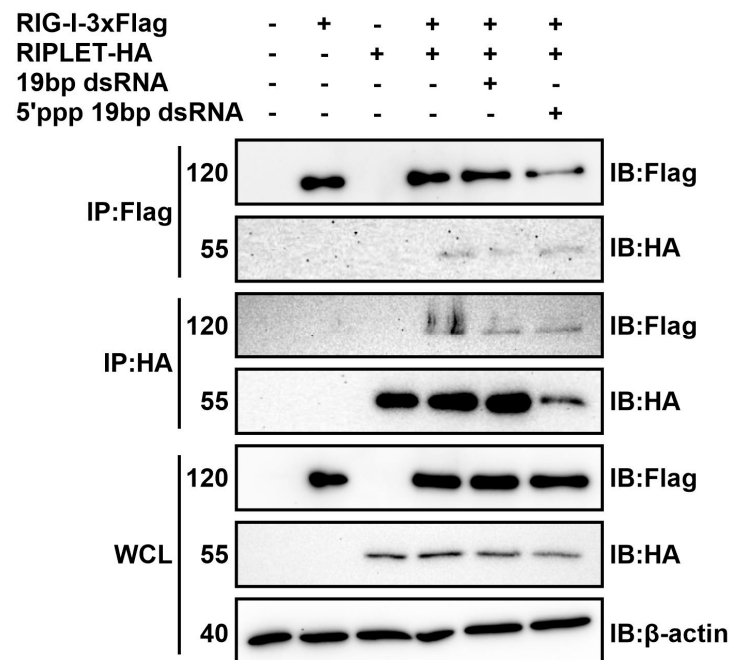


Figure 4. Co-immunoprecipitation of RIG-I with RIPLET in the presence of different ligands. DF-1 cells in 6-well plates were transfected with 1 µg of construct expressing each of the indicated tagged duck proteins. Six treatments were tested: EV (empty vector), RIG-I-3xFlag only, RIPLET-HA only, and three co-expressing both. Six hours later, samples were transfected with no ligand or transfected with 1 µg of dsRNA and 5'ppp dsRNA. After lysing the cells, whole-cell lysates (WCL) were subjected to anti-Flag and anti-HA immunoprecipitation (IP), followed by immunoblotting (WB) with anti-Flag and anti-HA antibodies. Raw data images of Western blot replicates are presented in Supplementary Data.

3.5. Inhibition of Duck RIPLET-Activated RIG-I by NS1

To test whether NS1 can inhibit duck RIPLET-activated RIG-I signaling, we chose WT-PR8-NS1 for our dual luciferase assays to measure chicken IFN-β promoter activity, as it has naturally occurring mutations that prevent it from binding to CPSF30 [38] but do not prevent it from binding to human or duck TRIM25 [37]. To determine whether NS1 can also bind dsRNA or RIPLET to disrupt the ability of RIG-I to stimulate IFN signaling, we compared 19bp 5'ppp dsRNA with no ligand. Interestingly, WT-PR8-NS1 inhibited the duck RIPLET activation of RIG-I, shown by a decrease in IFN-β promoter activation in the presence or absence of ligand (Figure 5A). To determine whether NS1 from other strains is also capable of inhibition, we tested WT-NS1 from four avian strains: BC500, CA431, D4AT, and VN1203. All NS1 proteins from avian strains were able to inhibit the IFN-β promoter response even more than WT-PR8 (Figure 5B). We observed that the co-expression of WT-NS1 from avian strains decreased the expression of all proteins, including RIG-I and RIPLET and NS1 (Figure 5D).

To determine whether this inhibition was due to NS1 binding to CPSF30, we used G184R mutants of NS1 known to eliminate CPSF30 binding, which otherwise would result in the overall and non-specific downregulation of protein synthesis. We also tested PR8-R38A mutant NS1, which is a loss-of-function mutant that was made to abolish interaction with TRIM25 [37], to determine whether it could also influence the RIPLET-mediated RIG-I signaling. None of the NS1 mutants decreased chicken IFN-β promoter activity (Figure 5C). Co-expression of these mutant NS1 strains with RIG-I and RIPLET also confirmed that the G184R mutation disrupted the interaction with CPSF30, as the expression of all proteins was recovered (Figure 5E). Notably, PR8-R38A NS1 and PR8-G184R NS1 were also unable to reduce signaling, as shown by no decrease in IFN-β promoter activity.

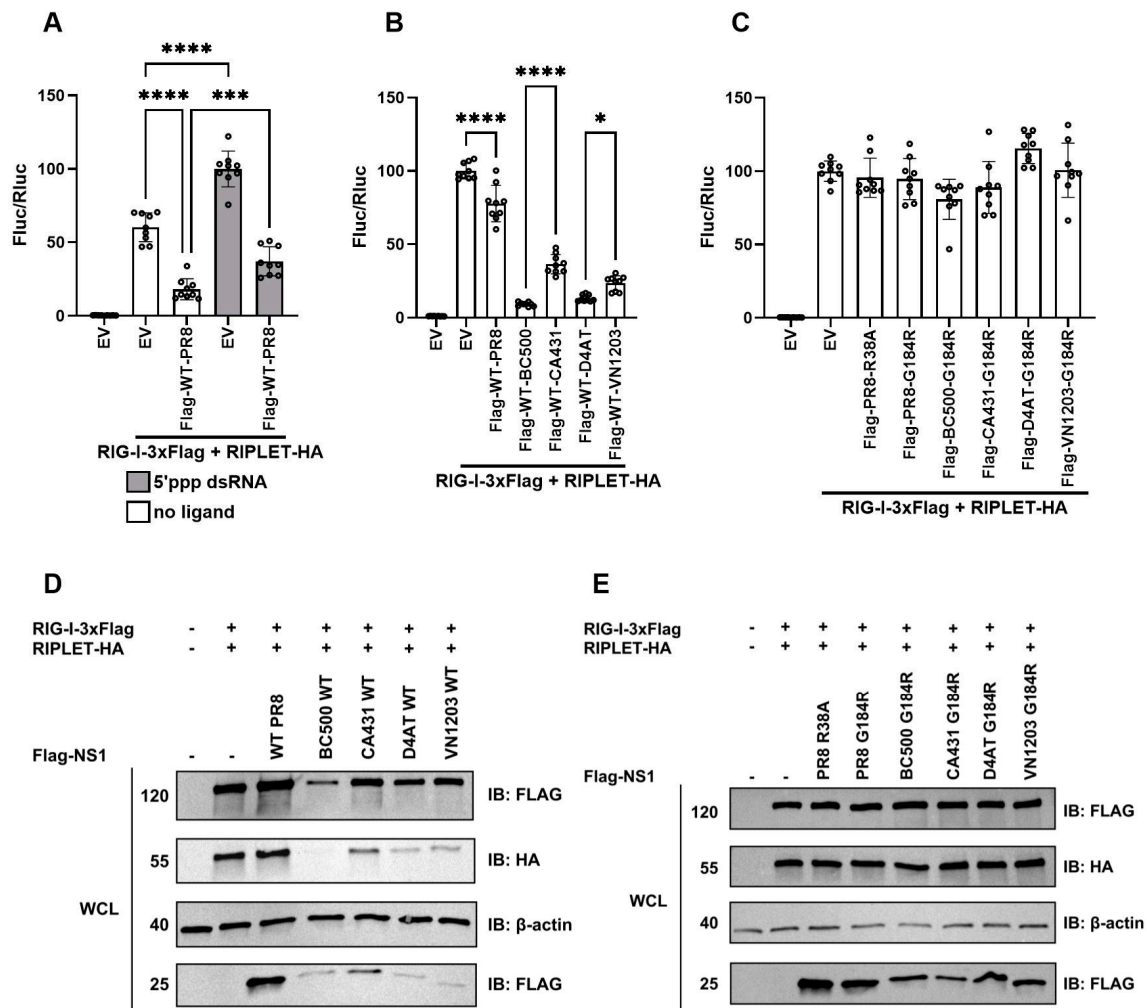


Figure 5. NS1 inhibition of duck RIPLET-activated RIG-I signaling. (A) Dual luciferase assay showing relative chicken IFN- β promoter activity inhibited by WT-PR8-NS1, in the absence or presence of 19 bp 5'ppp dsRNA, suggesting the disruption of RIPLET activation of RIG-I. (B) IFN- β promoter activity in the presence of WT-NS1 proteins from various avian influenza strains (BC500, CA431, D4AT, VN1203) compared with PR8, with BC500 and D4AT showing the greatest decrease in IFN promoter activation. (C) IFN- β promoter activity in the presence of G184R-NS1 mutants from various influenza strains PR8, BC500, CA431, D4AT, VN1203. (D) Co-expression of duck RIG-I and RIPLET with Flag-tagged WT-NS1 from different strains. (E) Co-expression of duck RIG-I and RIPLET with Flag-tagged mutant strains of NS1. Statistical significance was assessed using Tukey's multiple comparison under one-way analysis of variance (ANOVA) (* = $p < 0.01$, *** = $p < 0.0001$, **** = $p < 0.00001$).

3.6. Colocalization Analysis of Duck RIPLET with BC500 and VN1203 NS1

To determine the extent of colocalization between NS1 variants and duck RIPLET, we performed confocal imaging for the two NS1 proteins (BC500 and VN1203) and calculated Pearson's correlation coefficient. We observed no difference in the localization of wildtype and G184R versions of NS1. The results indicated a moderate degree of colocalization across all tested NS1 strains, with no statistically significant differences observed among the variants (Figure 6A,C). In the absence of RIPLET, NS1 showed similar cellular distribution (Figure 6B). Manders' coefficient M1 values suggested that a higher proportion of duck RIPLET overlapped with WT-BC500 NS1, whereas M2 values showed no significant differences among the NS1 variants, indicating that the degree to which NS1 overlaps with RIPLET remains comparable across conditions (Figure 6C). Consistent with

this moderate colocalization, we were sometimes able to pull down RIPLET with BC500 NS1 (Supplementary Figure S2); however, this was not reproducible.

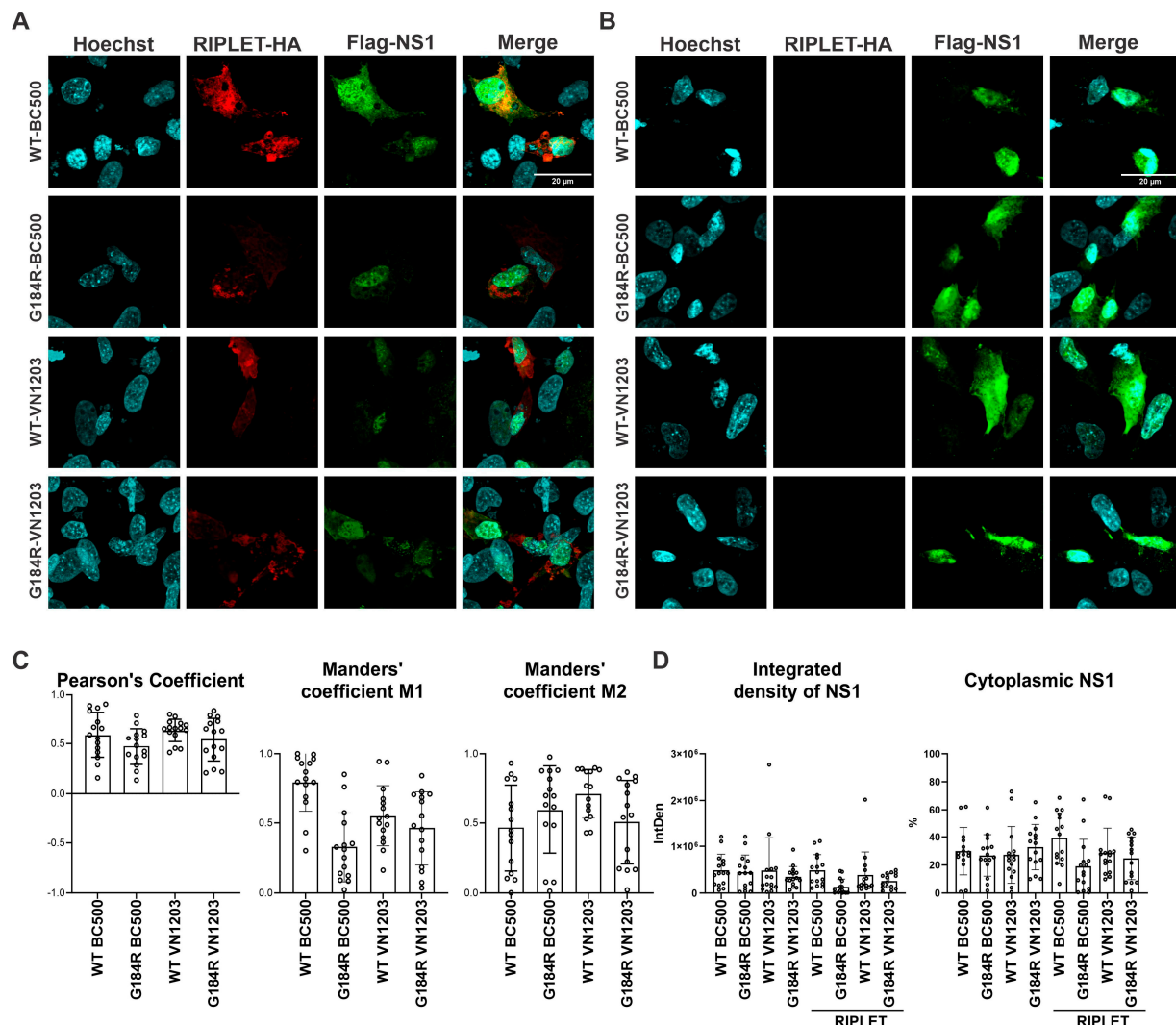


Figure 6. Subcellular localizations of duck RIPLET and BC500-NS1 or VN1203-NS1. The nucleus is shown in cyan, duck RIPLET in red and NS1 in green. (A) Representative images showing subcellular distribution of NS1 when co-expressed with duck RIPLET. (B) Representative images of NS1 without duck RIPLET (C). Colocalization of NS1 with duck RIPLET shown by Pearson's correlation coefficient and Manders' colocalization coefficient, expression of NS1 measured by IntDen and the percentage of cytoplasmic NS1 in the presence or absence of duck RIPLET. $n = 15$ cells/group. Statistical significance was assessed using Tukey's multiple comparison under one-way analysis of variance (ANOVA).

To investigate NS1 expression levels and its localization when co-expressed with duck RIPLET, we measured integrated density (IntDen = Area $\times$ Mean Intensity) as an indicator of protein expression. The total IntDen values remained unchanged when NS1 was co-expressed with duck RIPLET, indicating that RIPLET does not significantly influence NS1 expression levels (Figure 6D). To assess NS1 localization, we calculated the percentage of cytoplasmic NS1 by measuring IntDen separately in the cytoplasm and nucleus and determining their ratio. NS1 remained primarily localized in the nucleus regardless of RIPLET co-expression (Figure 6D).

4. Discussion

Duck RIPLET co-transfected with duck RIG-I can significantly increase IFN signaling as measured by IFN- β promoter activity in DF-1 cells, particularly upon stimulation with 19 bp 5'ppp-dsRNA. DF-1 cells expressing duck RIPLET and RIG-I show a significant upregulation of several key antiviral genes and a reduction in the influenza M1 gene mRNA transcripts when infected with LPAI (H6N2). Duck RIPLET and RIG-I colocalized in DF-1 cells and co-immunoprecipitated regardless of the presence of tested RNA ligands. These findings combined suggest that duck RIPLET is involved in the activation of duck RIG-I and downstream signaling. Finally, the NS1 from PR8, and several avian IAV strains of low and high pathogenicity, can inhibit the duck RIPLET activation of RIG-I. Mutant G184R NS1 proteins were created to disrupt interaction with CPSF30; however, they fail to inhibit the RIPLET activation of RIG-I.

In concordance with other studies in human and murine cells [10,13,49], we show that the RING domain in the duck RIPLET is also important in facilitating RIG-I activation, as duck Δ RING-RIPLET failed to enhance IFN- β promoter activity when co-expressed with duck RIG-I. However, Δ RING-RIPLET resulted in a slight upregulation of antiviral genes when transfected at higher concentrations, albeit to a lesser extent compared with full-length RIPLET. While all three replicates of RIG-I and RIPLET showed the downregulation of the influenza M1 transcript, in one out of three experiments we observed significant downregulation when co-expressing the duck Δ RING-RIPLET and RIG-I. Since the RING domain is the catalytic domain with ubiquitin ligase activity, Δ RING-RIPLET may have a ubiquitin independent function, similar to human RIPLET of cross-bridging RIG-I filaments by forming homodimers through its coiled-coiled domains [13]. Based on AlphaFold predictions, the RING domain was also suggested to be involved in the dimerization of RIPLET [42]. However, further studies are needed to confirm possible RING-independent functions of duck RIPLET or whether Δ RING-RIPLET partners with another E3-ubiquitin ligase.

Interestingly, overexpressed duck RIG-I and RIPLET even in the absence of ligand are sufficient to initiate downstream signaling cascades leading to IFN- β promoter activation. Studies in mammalian cells have shown that RIG-I requires ligand binding to undergo conformational changes that expose the CARD domains, essential for downstream signaling [42,50,51]. Human RIPLET was found to associate with RIG-I regardless of RNA presence; however, detectable signaling requires some ligand [42]. An early study by Saito et al. [52] demonstrated that expressing the 2CARDS of RIG-I alone could trigger an IFN response even in the absence of viral RNA, highlighting the constitutive activity of the isolated 2CARD. Our findings show that, at low concentrations, the activity of full-length duck RIG-I matched that of 2CARD when co-expressed with a high concentration of RIPLET. The ligand-independent activation observed in our study may be due to a conformational change in duck RIG-I induced by RIPLET exposing the 2CARD, when both proteins are overexpressed in the cell, mimicking the effect of viral ligand binding.

Duck RIPLET is a potent activator of RIG-I-dependent IFN- β promoter activity, and we do not observe the activation of full-length RIG-I by TRIM25 alone. However, both TRIM25 and RIPLET when transfected together significantly enhance RIG-I downstream signaling compared with RIPLET alone. Studies of human RIG-I support a role for TRIM25 ubiquitination of RIG-I CARD domains at lysine 172 [8,53,54], although RIPLET may also fulfill this role [10,13,14,49]. It is not known whether specific residues on duck RIG-I are ubiquitinated by RIPLET, or whether unanchored ubiquitin is made. Duck RIG-I CARD lacks the K172 residue important for human RIG-I activation, and the modification of conserved lysine does not abrogate activity, suggesting that duck RIG-I CARD is not activated by attached ubiquitin [47]. Recently, Li et al. [55] showed a structural role for TRIM25,

which may augment the RIG-I oligomerization required for signaling. Interestingly, our findings reveal a dual regulatory role of TRIM25 on the 2CARD domains of RIG-I. At lower concentrations of 2CARD, either TRIM25 or RIPLET enhance the IFN- β response, consistent with previous findings that TRIM25-mediated ubiquitination of 2CARD domains can enhance their activation [8,37,47]. However, at higher concentrations of 2CARD, TRIM25 appears to exert a negative effect on IFN- β production, whereas RIPLET has no such negative effect, remaining neutral. Different mammalian TRIM25 knockout cell lines exhibited significantly higher RIG-I-dependent downstream signaling in the absence of TRIM25, suggesting that TRIM25 might be a negative regulator [13]. This balancing act by TRIM25 may be crucial in maintaining homeostasis during viral infections, preventing excessive inflammation and potential autoimmune reactions.

The distribution pattern of duck RIG-I closely resembles that of duck RIPLET expressed in DF-1 cells. Surprisingly, the introduction of 5'ppp dsRNA did not significantly alter the subcellular distributions of duck RIG-I and RIPLET, which differs from the localization of human proteins by Oshiumi et al. [53]. They report that, in HeLa cells, ectopically expressed human RIPLET-HA colocalizes with endogenous RIG-I upon infection with VSV [53]. This static distribution of duck RIG-I and RIPLET in DF-1 cells might be due to the overexpression of duck RIG-I and RIPLET that mimics the effect of viral ligand binding and thus they are not redistributing due to the additional 5'ppp dsRNA ligand. Also, duck RIG-I did not colocalize with mitochondria in the presence or absence of the 5'ppp dsRNA ligand. Previous studies showed that a proportion of RIG-I associated with the mitochondrial activator of antiviral signaling (MAVS) upon infection with SeV using a proximity labeling assay in human HeLa cells [56]. It is noteworthy that RIG-I might transiently associate with MAVS in mitochondria during certain stages of the antiviral response [56]. In our colocalization study we used tagged proteins and antibodies, which is not as sensitive as proximity labeling assays.

Our results indicate that duck RIG-I and RIPLET can be co-immunoprecipitated regardless of ligand presence. We tested whether 19 bp dsRNA or 19 bp 5'ppp dsRNA could increase the amount of proteins interacting; however, the presence of ligand did not enhance the amount of protein pulled down. Human RIPLET can bind RIG-I without a ligand with moderate affinity, but the affinity is significantly higher when RIG-I is bound to 24 bp 5'ppp dsRNA [42]. However, the interaction is dependent on the length of ligand, as binding occurred between human RIG-I and RIPLET in the presence of 5'ppp dsRNA ligand at least 21 bp in length, while a 15 bp 5'ppp dsRNA did not result in protein binding [13]. Both colocalization and pulldown of duck RIPLET and RIG-I did not increase in the presence of 19 bp 5'ppp-dsRNA ligand, which may be due to suboptimal ligand length.

Our experiments show that WT-PR8-NS1 inhibits IFN- β promoter activity regardless of the presence of 19 bp 5'ppp dsRNA, indicating that NS1 can disrupt the RIPLET activation of RIG-I independent of dsRNA sequestration. WT-PR8-NS1 decreases IFN promoter activity independent of CPSF30 binding [37,38]. Further investigation using NS1 from four other strains capable of binding CPSF30 (BC500, CA431, D4AT, and VN1203) revealed that these variants more effectively inhibit the IFN- β promoter activity compared with WT-PR8, although some of this must be attributed to CPSF30 binding. Although virulence factors other than NS1 are also involved, we do see a reduction in interferon-stimulated gene expression in H6N2 (CA431)-infected DF-1 cells expressing RIG-I and RIPLET. Further, BC500 NS1 and D4AT NS1 showed the most inhibition of the IFN- β promoter activity. The inhibition also correlated with viral challenge experiments in birds when comparing the two HPAI strains, D4AT, isolated from a duck [57], and VN1203, obtained from a fatal

human case [58]. D4AT exhibited greater pathogenicity in ducks than VN1203 [59], which is consistent with the relative inhibition of NS1 from these two strains.

Our experiments to test the inhibition of RIPLET-mediated RIG-I signaling using previously constructed G184R mutant strains of NS1 designed to eliminate CPSF30 binding [37] show that they fail to decrease chicken IFN- β promoter activity. This finding suggests that the G184 residue is critical for the inhibitory function of NS1. It is noteworthy that the G184R mutation of PR8 abrogated the inhibitory activity of this NS1 protein, even though the WT-PR8-NS1 does not bind CPSF30, strongly suggesting that this modification interferes with the NS1 interaction with RIPLET. Similarly, the R38A mutant, originally designed to abolish TRIM25 interaction (35), also failed to inhibit the RIPLET-mediated activation of RIG-I. This observation implies that the R38 residue may play a broader role, potentially including its interaction with RIPLET. Earlier studies have shown that R38A/K41A mutants of NS1 were deficient in dsRNA binding [60,61], RIG-I binding [30] and RIPLET binding [31].

Our findings indicate a moderate colocalization between BC500-NS1 or VN1203-NS1 and duck RIPLET, with no statistically significant difference between the different NS1 proteins, as determined by Pearson's correlation coefficient and Manders' coefficients. The subcellular localization analysis demonstrated that the co-expression of duck RIPLET with either BC500-NS1 or VN1203-NS1 in DF-1 cells did not alter the predominantly nuclear localization of NS1. This observation differs from the findings of Rajsbaum et al. [31], who reported that the co-expression of mouse RIPLET with PR8-NS1 in HeLa cells led to a significant relocation of 90% of the NS1 from the nucleus to the cytoplasm. The difference may be attributed to the use of avian strains and cells, as our study examined duck RIPLET and BC500-NS1/VN1203-NS1 in DF-1 cells.

In conclusion, our study of duck RIPLET and RIG-I demonstrates the importance of these proteins in avian immune responses and antiviral defense mechanisms in the natural reservoir of influenza A viruses. We provide insight into the cellular distribution and molecular interactions of duck RIPLET and RIG-I. Finally, we show that influenza NS1 may target duck RIPLET to inhibit the activation of RIG-I.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v18020264/s1>, Figure S1: Guide linking original gels to final western blots; Figure S2: Co-immunoprecipitation of duck RIPLET with BC500 NS1; ZIP file S1: Original (raw) gel images.

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Abbreviations

The following abbreviations are used in this manuscript:

AIV	Avian Influenza Virus
CPSF30	Cleavage and Polyadenylation Specificity Factor 30
DF-1	Douglas Foster-1 (chicken embryo fibroblast cell line)
dsRNA	Double-Stranded RNA
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A Virus
MAVS	Mitochondrial Antiviral Signaling
NS1	Non-Structural Protein 1
RIG-I	Retinoic Acid-Inducible Gene I
RING	Really Interesting New Gene
RIPLET	RING Finger Protein Leading to RIG-I Activation

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