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Functional characterization of goose IRF1 in IFN induction and anti-NDV infection

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Abstract

Interferon regulatory factors (IRFs) play a key role in many aspects of immune response, and IRF1, IRF3, and IRF7 are positive regulators of IFN induction in mammals. However, IRF3, as the most critical regulatory factor in mammals, is naturally absent in birds, which attracts us to study the functions of other members of the avian IRF family. In the present study, we cloned goose IRF1 (GoIRF1) and conducted a series of bioinformatics analyses to compare the protein homology of GoIRF1 with that of IRF1 in other species. The overexpression of GoIRF1 in DF-1 cells induced the activation of IFN- β , and this activation is independent of the dosage of the transfected GoIRF1 plasmids. The overexpression of GoIRF1 in goose embryonic fibroblasts (GEFs) induced the expression of IFNs, proinflammatory cytokines, and IFN-stimulated genes (ISGs); it also inhibited the replication of green fluorescent protein (GFP)-tagged Newcastle disease virus (NDV) (NDV-GFP) and GFP-tagged vesicular stomatitis virus (VSV) (VSV-GFP). Our results suggest that GoIRF1 is an important regulator of IFNs, proinflammatory cytokines, and ISGs and plays a role in antiviral innate immunity in geese.

Keywords: Goose, IRF1, IFN, innate immunity, NDV

Introduction

As a host's first line of defense against exogenous insults, the innate immune system, which is broadly conserved in vertebrates, plays an important role in the host's resistance to infections such as viruses, bacteria, and parasites. Pathogen-associated molecular patterns (PAMPs) are evolutionarily conserved molecular vital structures in pathogens, such as lipopolysaccharides, glycoproteins, proteoglycans, and nucleic acid motifs (RNA and DNA) [1]. Pattern recognition receptors (PRRs) located on the cell membrane or in the cell, which include nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), toll-like receptors (TLRs), retinoic acid-inducible

gene-I (RIG-I)-like helicases (RLHs), and several cytoplasmic DNA sensors, are proteins encoded by host germline genes that can recognize PAMPs [2–6]. The innate immune system is particularly critical for the host to detect invading pathogens and activate subsequent adaptive immunity [7]. The triggering of this system depends on the host's PRRs to specifically recognize and bind to PAMPs and then activate related receptor-dependent signaling cascades to induce cytokine production [2]. Exogenous insults, such as bacteria, viruses, and parasites, are quickly recognized by the host's innate immune system, which then induces several immune mechanisms to resist invasion, including an interferon (IFN)-mediated antiviral response and an interleukin (IL)-mediated pro-inflammatory response [8, 9]. The type I IFN response is an effective defense of the host against viral infection [10].

Type I IFN is an important class of antiviral cytokines, including IFN- α , IFN- β , IFN- κ , and so on [11]. The

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induction of type I IFN is mainly controlled at the gene transcriptional level, and the interferon regulatory factors (IRFs) play central roles [12]. IRFs are transcription factors in IFN signaling pathways that play an important role in immune responses against bacterial or viral invasions [13]. So far, 11 members of the IRF family, IRF1 to IRF11, have been identified in vertebrates and 9 of them are expressed in mammalian cells (IRF1–IRF9) [14]. Compared with mammals, birds seem to naturally lack IRF3 and IRF9 [15], but the unique IRF10 can be found in them [16]. More importantly, it is widely accepted that a closely related IRF7, considered to be the major IRF in birds, complements the functions of IRF3, considered to be the critical IRF in mammals, and that other IRFs such as IRF1 may also compensate the functions of IRF3 to a certain extent [17–20]. In addition, both IRF10 and IRF11 have been identified in fishes [21]. The amino terminus of all IRFs contains a well-conserved DNA binding domain (DBD), which is characterized by five well-conserved tryptophan (Trp, W)-rich repeats, and the DBD contains a helix-turn-helix domain that recognizes specific DNA sequences [22]. The C-terminal amino acid of IRFs is less well conserved and contains an IRF-associated domain (IAD), which confers each IRF a unique function [23].

IRF1 is the first IRF family member found to be involved in the regulation of IFN [24]. Conservatively, the first 115 amino acids of the N-terminal of IRF1 contain a DBD with five highly conserved tryptophan-rich repeats that determines its transcriptional activity [25]. Mammalian IRF1 binds to the hexanucleotide unit in the positive regulatory domain 1 (PRD1) of the IFN- β promoter to regulate IFN expression [26]. Crystallographic studies further revealed that the 5'-GAAA-3' sequence of PRD1 is the core sequence recognized by the DBD of IRF1 [27]. The conserved tryptophan cluster within the DBD was found to be critical in recognizing the core sequence. Three of the five tryptophan residues (W11, W38, and W58) fix the helix-turn-helix motif within the DBD to the major groove of the DNA, forming contact with the sugar-phosphate backbone and allowing the other four conserved amino acids of the DBD [Arginine (Arg, R) 82, Cysteine (Cys, C) 83, Asparagine (Asn, N) 86 and Serine (Ser, S) 87] direct contact to the core sequence [27]. Mammalian IRF1 plays an important regulatory role in antiviral innate immunity. After a viral infection, IRF1, which is significantly upregulated and expressed in cells, inhibits virus replication by regulating the production of IFN [28, 29]. IRF1 interacts with myeloid differentiation primary-response protein 88 (MyD88) to regulate the TLRs (TLR2, TLR3, TLR4, TLR7, TLR8)-dependent signal cascade and promote the production of IFN or IL to resist infection [30]. In addition, IRF1 has also been confirmed to be involved in the regulation of physiological or

pathological processes, such as tumor immune surveillance, proinflammatory injury, immune system development, and autoimmune diseases [31].

The immune systems of birds and mammals are quite different, and birds lack several key immune genes [32]. IRF3, as one of the most critical IRFs in mammals, is naturally absent in poultry [33, 34]. Whether its absence leads to avian immune system deficiency remains to be studied. IRF1 plays a role in mammalian antiviral innate immunity, but its function in poultry needs to be more comprehensively studied. It is not clear whether avian IRF1 can compensate for the missing IRF3 function. Our previous study demonstrated that chicken IRF1 inhibits the replication of avian influenza virus (AIV) and Newcastle disease virus (NDV) by regulating the production of IFN- β and ISGs [35]. Duck IRF1 regulates the production of IFN- β by interacting with MyD88, effectively inhibiting virus replication [36]. However, the characteristics and functions of IRF1 in geese have not yet been elucidated. Chickens, ducks, and geese have different levels of resistance to AIV and NDV. As a key regulatory factor of the immune system, the functional difference of IRF1 may be the reason for the difference in antiviral levels of different birds. Therefore, it is necessary to conduct in-depth research on the function of GoIRF1 in antiviral innate immunity.

In the present study, GoIRF1 was identified and cloned from a cDNA spleen sample, and the function of IRF1 in innate immunity in geese was explored. We investigated the function of GoIRF1 in RNA virus infection and demonstrate that GoIRF1 plays a role in limiting replication and infection by NDV. Our results suggest that GoIRF1 is an important regulator of IFNs, proinflammatory cytokines, and ISGs and is involved in antiviral innate immunity in geese. These findings contribute to a more systematic understanding of the bird's IFN-regulated signaling pathway and innate immune system and provide reference data about the general and individual characteristics of the innate immune system in birds and mammals.

Materials and methods

Cells and viruses

DF-1 is a chicken embryonic fibroblast cell line from East Lansing strain eggs. Goose embryonic fibroblasts (GEFs) were prepared from 15-day-old embryonated goose eggs. The DF-1 cells and GEFs were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Corning, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin–streptomycin (Gibco). All cells were incubated at 37 °C in a 5% carbon dioxide incubator. The NDV strain NSD14 was isolated from chickens at a farm in Shandong Province, China. GFP-tagged NDV

low virulent strain LaSota, named NDV-GFP and VSV-GFP, are stored in our laboratory. These three viruses were purified, propagated, and stored as described in our previous study [37].

Cloning and bioinformatics analysis of GoIRF1

Based on the predicted GoIRF1 sequence (XM_013175976.1) obtained from the National Center for Biotechnology Information (NCBI), the primers GoIRF1-F and GoIRF1-R (Table 1), which are located

outside the GoIRF1 open reading frame, were designed. The above primers and ApexHF HS DNA Polymerase FS Master Mix AG12202 (Accurate Biotechnology Co., Ltd., Hunan, China) were used to amplify GoIRF1 cDNA from a spleen sample via transcriptase polymerase chain reaction (PCR). The PCR product was ligated into a pTOPO-Blunt vector (Aidlab Biotech, Beijing, China) for sequencing, and positive colonies were sent to the Beijing Genomics Institute (Beijing, China) for sequencing. The amino acid sequence of GoIRF1 was aligned

Table 1 PCR primers used in the study

Target gene	Purpose	Name	Sequence of oligonucleotide (5'-3')	
GoIFN-α	qRT-PCR	qGoIFN-α F	CTCCAGCACCTCTTCGACAC	
		qGoIFN-α R	GTTGATGCCGAGGTGAAGGT	
GoIFN-γ	qRT-PCR	qGoIFN-γ F	ACATCAAAAACCTGTCTGAGCAGC	
		qGoIFN-γ R	AGGTTTGACAGGTCCACGAGG	
GoIFN-κ	qRT-PCR	qGoIFN-κ F	ACAGCAAAGAAAAGTGATTG	
		qGoIFN-κ R	GTTGGAAGATCTCTCAATGG	
GoIFN-λ	qRT-PCR	qGoIFN-λ F	GAGCTCTCGGTGCCCGACC	
		qGoIFN-λ R	CTCAGCGGCCACGCAGCCT	
GoIL-6	qRT-PCR	qGoIL-6 F	AGCAAAAAGTTGAGTCGCTGTGC	
		qGoIL-6 R	TAGCGAACAGCCCTCACGGT	
GoIL-8	qRT-PCR	qGoIL-8 F	GCTGTCTGGCTCTTCTCTGATT	
		qGoIL-8 R	GGGTCCAAGCACCTCTCTGTGG	
GoPKR	qRT-PCR	qGoPKR F	GCAACAGCAAAGACTGACGA	
		qGoPKR R	TGTTTGTGACCTCTGCCTTG	
GoOASL	qRT-PCR	qGoOASL F	CAGCGTGTGGTGGTTCTC	
		qGoOASL R	AACCAGACGATGACATACAC	
GoMx-1	qRT-PCR	qGoMx-1 F	TTCACAGCAATGGAAGGGA	
		qGoMx-1 R	ATTAGTGTCCGGTCTGGGA	
GoIRF1	qRT-PCR	qGoIRF1 F	TGAGAAAGACCCTGACCCCA	
		qGoIRF1 R	GCTGGAGCCTTTGTTGATGC	
	To obtain sequence	GoIRF1 F	CTCTGCTTCTGTACAGCCAC	
		GoIRF1 R	CTGCAGAAGCCAGGGGTTTAC	
		Construction of GoIRF1	pcDNA3.1-Flag <i>Eco</i> R I	tagtccagtggtggaattcATGCCGTCTCCAGAATGCG
	Construct truncated forms of GoIRF1		pcDNA3.1-Flag <i>Xho</i> I	gtcgtccttgtagtctctcagCAAGCCACAGGAGATGGTTTG
			GoIRF1 d1-10 aa F	gtgtggtggaattcatgTGGCTGGAATGCAG
			GoIRF1 d1-10 aa R	CATGAATCCACCACAC
			GoIRF1 d1-50 aa F	gtgtggtggaattcatgGATGCCTGCCTTTTC
			GoIRF1 d1-50 aa R	CATGAATCCACCACAC
			GoIRF1 d51-114 aa F	ctgggacatggagaaaTTGACAAAGGATCAG
			GoIRF1 d51-114 aa R	TTTCTCCATGTCCCAG
			GoIRF1 d120-220 aa F	GCTTCATCCTCGGAAG
			GoIRF1 d120-220 aa R	cttccgaggatgaagcCTGATCCTTTGTC
			GoIRF1 d201-317 aa F	gactggaggacgccgCTCGAGGACTACAAG
GoIRF1 d201-317 aa R	CGGCGTCTCCAGTC			
GoIRF1 d251-317 aa F	caggactggcacacgCTCGAGGACTACAAG			
GoIRF1 d251-317 aa R	CGTGTGCCAGTCCTG			

with other animal IRF1 proteins from chickens, humans, pigs, and mice using Clustal W and edited with ESPrpt 3.0 [38]. Sequence homology and a phylogenetic analysis of the IRF1 amino acid sequences were conducted using DNASTAR. A phylogenetic tree was constructed based on the IRF1 from 13 different species, including mammals, birds, and fish. Conserved domains in the GoIRF1 amino acid sequences were predicted using the simple modular architecture research tool (SMART) program [39]. Homology modeling for GoIRF1 was conducted using the online protein-modeling server SWISS-MODEL [40].

Plasmid construction

Flag-tagged GoIRF1 plasmids (pcDNA3.1-GoIRF1-Flag) were constructed by inserting the full-length GoIRF1 into the *Xho* I and *Eco*R I sites pcDNA3.1-Flag of the expression vector using a ClonExpress II One-Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). The primers used in the PCR are listed in Table 1. The truncated plasmids of GoIRF1, including deletion of amino acids 1 to 10 (d1-10 aa), d1-50 aa, d51-114 aa, d120-220 aa, d201-317 aa, and d251-317 aa, were constructed using a modified homologous recombination method and the primers listed in Table 1. The chicken IFN- β (chIFN- β) promoter luciferase reporter plasmids (pGL-chIFN- β -Luc), which contained -158 to +14 of the IFN- β promoter motif, were constructed as described in our previous study [41]. The Trelief™ 5 α Chemically Competent Cell (Tsingke Biological Technology, Beijing, China) was used for plasmid transformation.

Luciferase reporter assays

The DF-1 or GEF cells were plated in 24-well plates (NEST Biotechnology, Wuxi, China) and cultured to 95–100% confluence; they were then transiently transfected with reporter plasmid pGL-chIFN- β -Luc (0.12 μ g/well) and internal control Renilla luciferase (pRL-TK, 0.06 μ g/well), along with the indicated plasmids, using Nulen PlusTrans™ Transfection Reagent (Nulen, Shanghai, China). The cells were lysed 24 h after transfection, and luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. Renilla luciferase activity was used for normalization. All reporter assays were repeated at least three times.

RNA extraction and quantitative real-time PCR

After the RNA was extracted from GEFs using an HP Total RNA kit (Omega, USA), the RNA was reverse-transcribed to cDNA using a cDNA synthesis kit (Vazyme). Quantitative real-time PCR (qRT-PCR) tests were conducted using the primers listed in Table 1 and an ABI

7500 RT-PCR system. The qRT-PCR test was performed according to the manufacturer's instructions using a ChamQ™ SYBR® qPCR Master Mix (Vazyme). The conditions and data processing method for the qRT-PCR test were the same as in our previous study [41].

Western blot analysis

The DF-1 cells were plated in 12-well plates at 1×10^6 /1 mL and then transfected with empty plasmids or various expression plasmids. After 24 h, the transfected cells were washed twice with phosphate buffer saline (PBS) (Gibco) and then lysed with a cell lysis buffer (Beyotime, Shanghai, China) containing an InStab™ protease cocktail (Yeasen, Shanghai, China) and phenylmethylsulfonyl fluoride (PMSF) (Yeasen). The cell lysates were centrifuged at 13 000 rpm for 15 min to obtain the supernatant, and a $5 \times$ SDS-PAGE protein loading buffer (Yeasen) was added. The lysates were then boiled for 10 min. The proteins isolated from the cell lysates were separated via SDS-PAGE and analyzed using a Western blot. Images were obtained using the Tanon 5200 imaging system (Tanon, Shanghai, China), as described in our previous study [42].

Viral infection

The GEF cells were plated, washed twice with PBS (Gibco), and infected at 0.1 multiplicity of infection (MOI) with NSD14. The RNA from the cells, which was infected with the virus at different times, was then collected for quantitative real-time PCR (qRT-PCR). The GoIRF1-overexpressing and normal DF1 cells were infected at 0.1 MOI with NDV-GFP or VSV-GFP, and fluorescence was measured 24 h after infection using a fluorescence microscope.

Statistical analysis

The data were expressed as means \pm standard deviations. Significance was determined using a two-tailed independent Student's *t* test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Cloning and sequence analysis of GoIRF1

To better understand the role of GoIRF1 in innate immunity in geese, we cloned GoIRF1 and conducted a bioinformatics analysis. The open reading frame of GoIRF1 contains 954 bp and encodes 317 amino acid residues. The IRF1 amino acid sequences in chickens (*Gallus gallus*, NP_990746.1), humans (*Homo sapiens*, NP_002189.1), mice (*Mus musculus*, CAJ18442.1) and pigs (*Sus scrofa*, NP_001090882.1) are 90.1%, 62.2%, 60.14%, and 59.6% identical to those in geese, respectively (Figure 1A). The protein domains of GoIRF1 were predicted using SMART. The results show that there is an IRF domain (DBD) at the N-terminal of GoIRF1 (aa 1-114) (Figure 1B). The DBD of

IRF1 in geese and mammals is highly conserved. In particular, the elements (W11, W38, W58, R82, C83, N86, S87) that mediate 5'-GAAA-3' sequence recognition are well preserved among different species (Figure 1C).

Phylogenetic tree analyses and the three-dimensional structure of GoIRF1

The amino acid sequence homologies of different animals were conducted using MegAlign; the results are shown in Figure 2A. A phylogenetic tree was developed based on multiple alignments of IRF1 from various species, including mammals, birds, and fish (Figure 2B). The resulting phylogenetic tree consists of three major branches. The IRF1 protein sequences of geese, chickens (NP_990746.1), and ducks (XP_027324327.2) belong to one subgroup. The IRF1 of mammals, including cattle (NP_001178190.1), sheep (NP_001009751.1), pigs (NP_001090882.1), dogs (XP_003639416.3), horses (XP_005599497.1), cats (XP_003980801.1), humans (NP_002189.1), monkeys (XP_012300198.1), and mice (CAJ18442.1), belongs to another subgroup. IRF1 sequences from zebrafish (NP_991310.1) belong to a third subgroup. These categorizations reflect the genetic relationships among these species. The predicted three-dimensional structures of GoIRF1 are shown in Figure 2C.

Upregulation of GoIRF1 expression in response to viral infection

In chickens and some mammals, IRF1 is involved in type-I IFN-mediated antiviral innate immune response. However, the role of goose IRF1 in antiviral response remains unknown. For the host, upregulating the expression of certain immune-related genes is an important strategy in infection resistance. To determine whether GoIRF1 could induce an antiviral response to infection with NDV, we conducted a preliminary analysis of the expression of GoIRF1, of some cytokines, and of the IFN-stimulated genes in GEF cells following infection with NDV. The results show that the mRNA levels of GoIRF1 were significantly upregulated during viral infection (Figure 3A). The mRNA levels of IFNs (IFN- α , IFN- κ , IFN- γ , and IFN- λ) (Figures 3B–E), IL6 (Figure 3F), and IFN-stimulated genes (ISGs) (Mx-1, PKR, and OASL) (Figures 3G–I) were significantly upregulated as well. These upregulations play a key role in cell resistance to exogenous insults.

GoIRF1 involvement in the regulation of IFNs, proinflammatory cytokines, and ISGs

IRF1 plays an important role in mammalian antiviral innate immunity, but its function in geese is unknown. To investigate whether GoIRF1 is also involved in the regulation of IFN production, DF-1 cells were cotransfected with GoIRF1

expression plasmids and with chIFN- β luciferase reporter plasmids. We found that the overexpression of GoIRF1 in DF-1 cells significantly activated the chIFN- β promoter (Figure 4A), and this activation is independent of the dosage of the transfected GoIRF1 plasmids (Figure 4B). To further confirm the role of GoIRF1 in IFN activation, we conducted luciferase assays with primary goose embryonic fibroblasts (GEFs). This test also shows that the overexpression of GoIRF1 in GEFs significantly activated the chIFN- β promoter (Figure 4C). To further explore the effect of GoIRF1 on the expression of innate immune genes, we conducted qRT-PCR tests after we transfected GoIRF1 or an empty vector into GEFs. The results show that the mRNA levels of IFNs (IFN- α and IFN- γ), proinflammatory cytokines (IL-6 and IL-8), and IFN-stimulated genes (PKR, OASL, and Mx-1) increased significantly when GoIRF1 was overexpressed (Figures 4D–F). In the presence of overexpressed GoIRF1, the expression levels of IFN- α , IFN- γ , IL-6, IL-8, PKR, OASL, and Mx-1 were 2.0, 10.2, 1.9, 1.6, 2.1, 3.7, and 6.2 times higher than in the controls (which were transfected with an empty vector), respectively. In addition, the overexpression of GoIRF1 in GEFs did not induce the upregulation of IFN- κ and IFN- λ .

Essential domains of GoIRF1 in IFN activation

The secondary structure predicted by the SMART program shows that there is an IRF domain (DBD) at the N-terminal of GoIRF1 (aa 1-114). A series of truncated forms of GoIRF1 were generated based on the secondary structure of GoIRF1. The ability of these forms to activate the IFN- β promoter was measured and compared to that of wild-type GoIRF1 using the dual luciferase reporter gene detection method (Figures 5A and B). As shown in Figure 5B, the deletion of 10 (d1-10 aa) or 50 (d1-50 aa) amino acids at the GoIRF1 N-terminal clearly decreased IFN- β promoter activity. Compared with the wild type, GoIRF1 has a significantly reduced ability to activate IFN- β when amino acids 51–114 are deleted. The deletion of amino acids 120–220 had little effect on IFN- β activation by GoIRF1. The C-terminal deletion mutants of GoIRF1 (d201-317 aa and d251-317 aa) also led to a significant degradation in IFN- β promoter activation. These results indicate that the first 114 amino acid sequences of GoIRF1 contain domains that are pivotal for inducing IFN- β promoter activity. In addition, the deletion of the C-terminal amino acid of GoIRF1 also led to a significant degradation in IFN- β promoter activation.

The in vitro antiviral role of GoIRF1

To test the antiviral effects of GoIRF1, the GoIRF1-overexpressing and normal DF-1 cells were infected with VSV-GFP or NDV-GFP, and fluorescence was measured with a fluorescence microscope. The fluorescence intensities of both VSV-GFP and NDV-GFP in the cells that

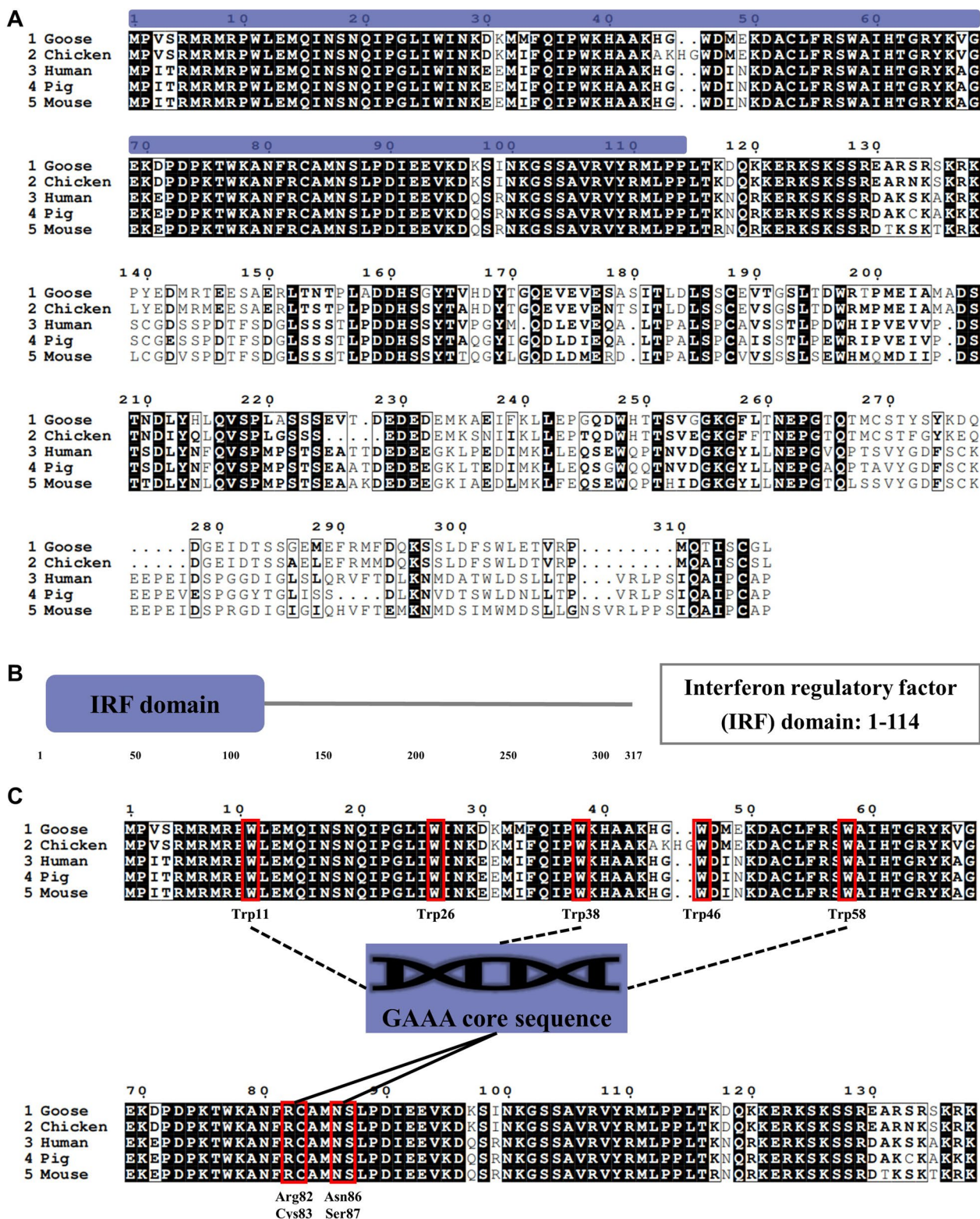
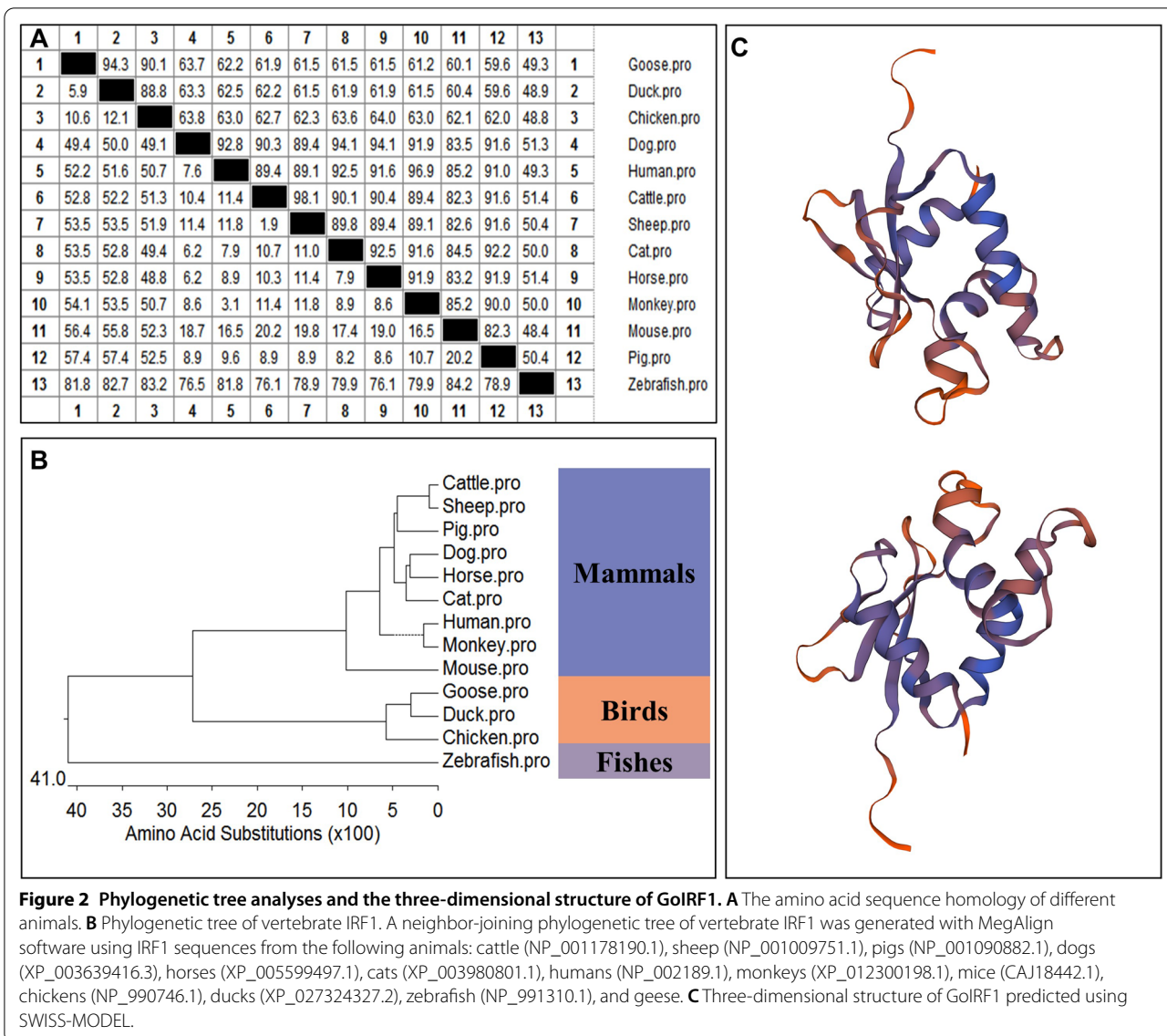


Figure 1 Sequence analysis of GolRF1. **A** Alignment of the amino acid sequence of GolRF1 with IRF1 proteins from humans (NP_002189.1), mice (CAJ18442.1), pigs (NP_001090882.1), and chickens (NP_990746.1). The amino acid sequences of different animals were aligned using Clustal W and edited with ESPrnt 3.0. The black shading indicates the identity of the amino acid, and the gray shading indicates similarity (50% threshold). **B** Protein domains of GolRF1 predicted by SMART. **C** The elements that mediate 5'-GAAA-3' sequence recognition in IRF1 are well preserved among different species.

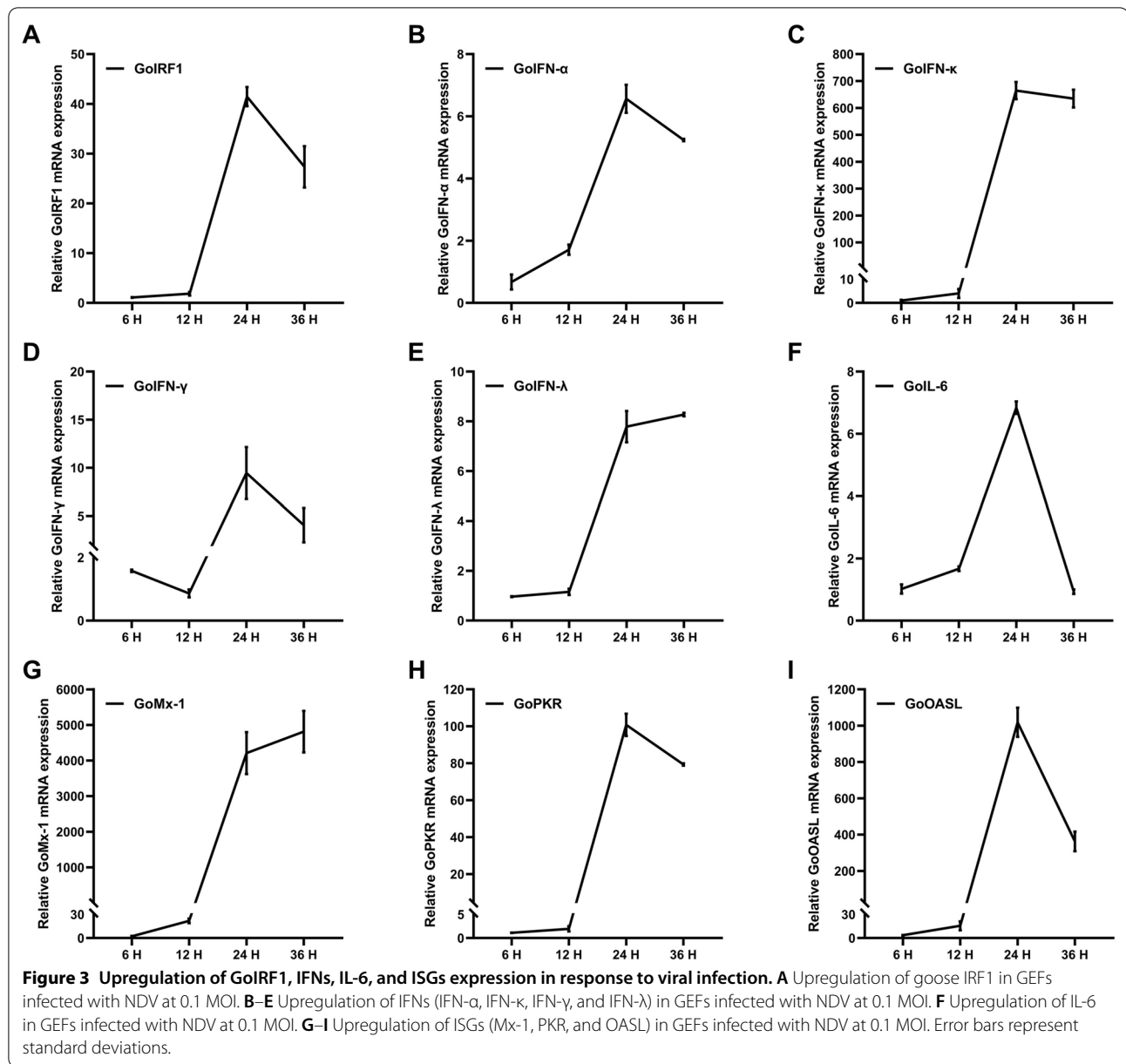


overexpressed GoIRF1 were significantly lower than those in the control cells 14 or 24 h after viral infection (Figures 6A and B). This result suggests that GoIRF1 overexpression in DF-1 cells suppresses VSV-GFP and NDV-GFP viral replication. The virus-infected cells were then lysed and collected for Western blot analysis. The GFP protein expression levels of NDV-GFP and VSV-GFP in the cells overexpressing GoIRF1 were significantly lower than those of the controls (which were transfected with an empty vector).

Discussion

Mammalian IRFs play a key role in many aspects of the immune response, and IRF1 is a positive mediator of IFN induction after PRRs recognize PAMPs [43]. Compared

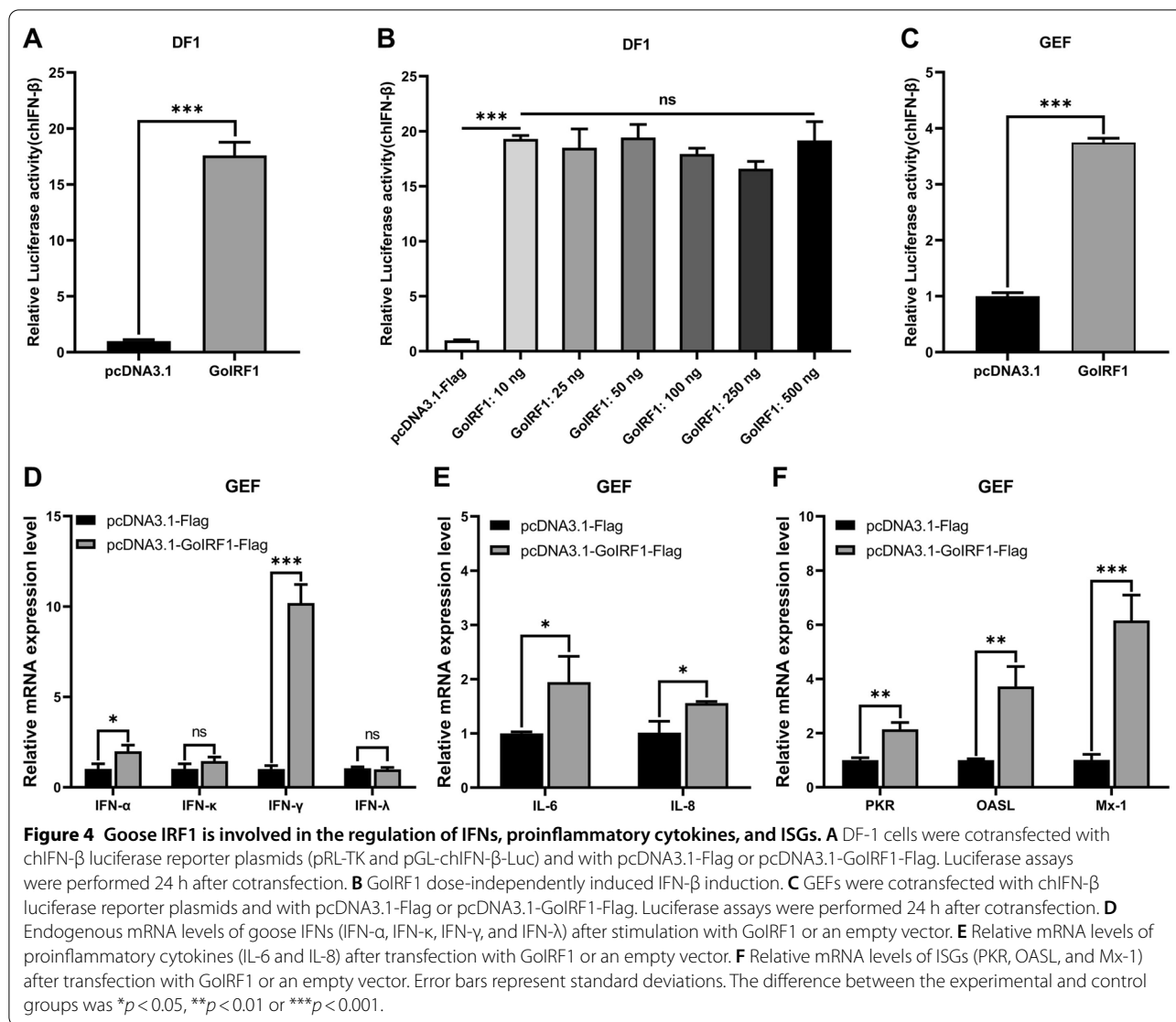
with mammals, birds have fewer immune genes, and IRF3, one of the most important IFN regulatory factors in mammals, is naturally absent in birds [15]. It is widely accepted that a closely related IRF7, considered to be the major IRF in birds, complements the functions of IRF3, and that other IRFs may compensate the function [18, 20]. It remains to be studied whether IRF3 deletion leads to deficiencies in the innate immune system of birds and whether IRF1 plays an immune regulatory function in IRF3-absent birds. Although the IRF1 of chickens and ducks has been shown to be an important regulatory factor involved in IFN regulation [35, 36], geese have shown more significant advantages in resisting AIV and NDV infections [44, 45], and IRF1 as a key IFN regulatory factor may be the cause of the difference in disease



resistance. A better understanding of the function of GoIRF1 may help explain these differences.

First, we cloned GoIRF1 and analyzed the amino acid sequences of IRF1 from different animals. The N-terminus of IRF1 in different animals contains a DBD, which is highly conserved among different species, while the C-terminus has poor homology, which determines the uniqueness of its function (Figure 1A). The conserved domains predicted by SMART show that there is an IRF domain (DBD) at the N-terminal of GoIRF1 (aa 1-114) (Figure 1B). Not surprisingly, the DBD of IRF1 in geese

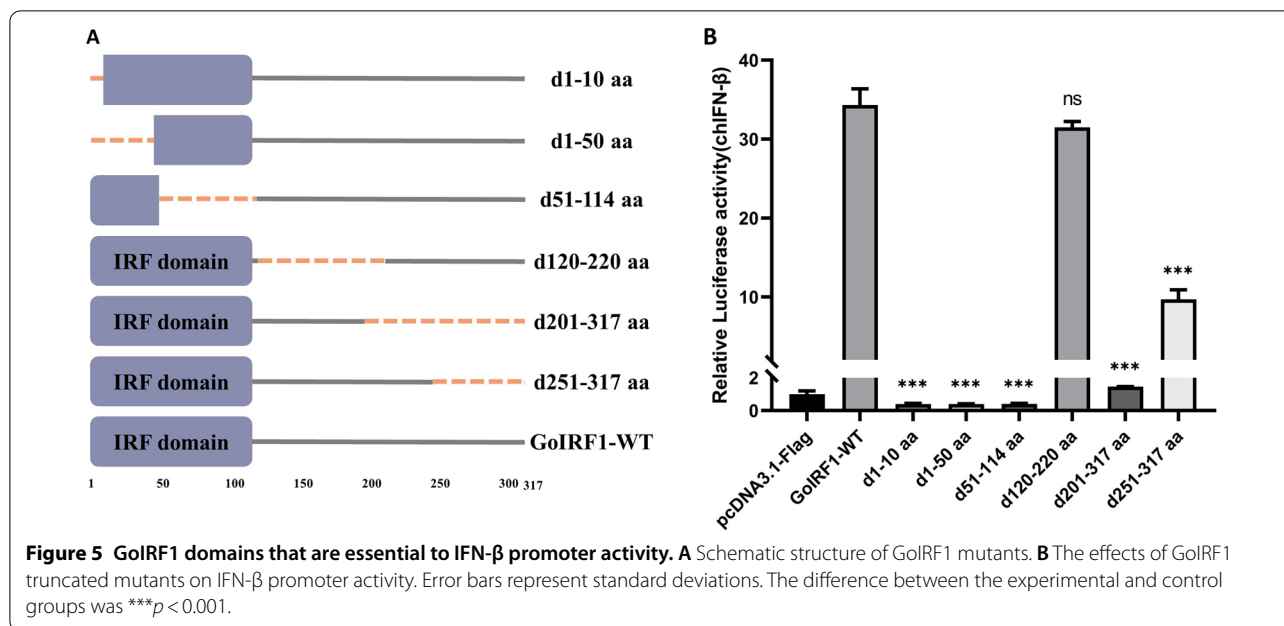
and mammals is highly conserved. In particular, the elements (W11, W38, W58, R82, C83, N86, and S87) that mediate 5'-GAAA-3' sequence recognition are well preserved among geese and mammals (Figure 1C). These conserved elements allow GoIRF1 to bind to the core sequence on the PRD1 of the IFN promoter to regulate IFN production [27]. The resulting phylogenetic tree consists of three major branches (Figures 2A and B). The IRF1 protein sequences of geese, ducks, and chickens belong to one subgroup. The IRF1 of mammals, including humans, monkeys, mice, cats, horses,



dogs, cattle, sheep, and pigs, belong to another subgroup. IRF1 sequences from zebrafish belong to a third subgroup. These results reflect the genetic relationships among these species. The predicted three-dimensional structures of GoIRF1 are shown in Figure 2C.

IRF1 was initially identified as a positive regulator that directly binds to the promoter of type I IFN genes, but subsequent studies have found that the absence of IRF1 in mice does not affect the induction of IFN-β [46]. Whether IRF1 is a necessary IFN regulatory factor was once in controversy. With the deepening of innate immunity research, IRF1 was found to interact with MyD88 to control the production of TLR9-dependent IFN-β in mouse myeloid dendritic cells [47]. IRF1 also plays a

positive regulatory role in the innate immune response to viral infection by enhancing the phosphorylation of IRF3 and the production of types I and III IFN triggered by viral infection [48]. For the host, upregulating the expression of certain immune-related genes is a required strategy in infection resistance. Mammalian IRF1 is upregulated by viral stimulation and resists viral infection by regulating IFNs and ISGs [48]. A qRT-PCR test shows that GoIRF1 mRNA was significantly upregulated in GEFs infected with NDV. Some key cytokines and ISGs that resist viral infection are also significantly upregulated, showing the same expression trend as IRF1. These results indicate that GoIRF1 may be involved in the regulation of antiviral innate immunity in geese.



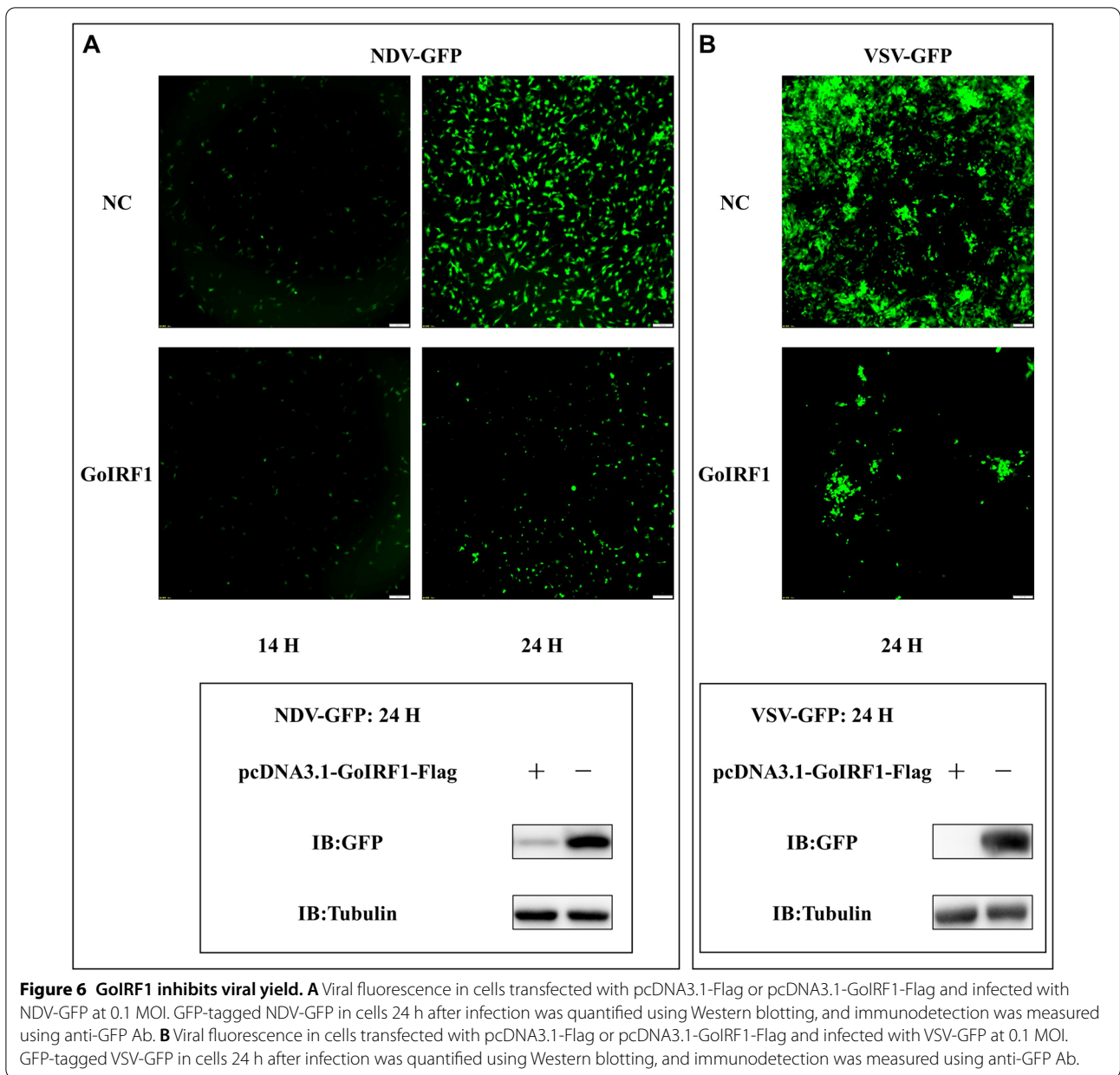
In cell experiments, we found that the overexpression of GoIRF1 in GEFs or DF-1 cells strongly induced the expression of chIFN-β, and this induction is independent of the amount of GoIRF1 plasmid transfected (Figures 4A–C). Other types of IFNs, including IFN-α and IFN-γ, were also activated by GoIRF1 overexpression, while IFN-κ and IFN-λ were not upregulated (Figure 4D). In addition, the overexpression of GoIRF1 in GEF cells significantly upregulated the expression of several pro-inflammatory cytokines (IL-6 and IL-8) and IFN-stimulated genes (PKR, OASL, and Mx-1) (Figures 4E and F). Our results indicate that GoIRF1 positively regulates the expression of IFNs, proinflammatory cytokines, and ISGs.

To identify the domain of GoIRF1 that is indispensable to IFN induction, a series of truncated forms of GoIRF1 were generated, and their abilities to induce IFN-β promoter activity were compared (Figure 5). The deletion of different fragments of the IRF domain (d1-10 aa, d1-50 aa, and d51-114 aa) leads to the loss of its function of activating IFN-β. These mutated IRF1 may be due to its inability to recognize the 5'-GAAA-3' core sequence on the IFN-β promoter, leading to the loss of its function, which indicates that the complete DBD is necessary for GoIRF1 to activate IFN-β. The deletion of amino acid fragments of different sizes at

the C-terminus of GoIRF1 also results in a significant decrease in its ability to activate the IFN-β promoter. In contrast, the deletion of amino acids 120–220 has little effect on the activation of the IFN-β promoter by GoIRF1. Our previous studies on the indispensable domains of chicken IRF1 to activate IFN also showed the same characteristics [35].

IRF1 plays an important role in the antiviral innate immunity of mammals, but the research on its antiviral function in birds is incomplete. In particular, the antiviral function of goose IRF1 is unknown. To further verify the immunomodulatory and antiviral activities of GoIRF1, we conducted a series of experiments to explore the effect of GoIRF1 on virus replication. The results suggest that GoIRF1 overexpression in DF-1 cells significantly suppresses the viral replication of VSV-GFP and NDV-GFP (Figure 6).

In brief, our results show that GoIRF1 is an important regulator of IFNs, proinflammatory cytokines, and ISGs and is involved in goose antiviral innate immunity. Our findings contribute to a more systematic understanding of the innate immune system of geese and could help explain the differences in the resistance of different birds to viral infection. Our results also provide reference data about the general and individual characteristics of innate immunity in birds and mammals.



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Authors' contributions

YC and JS designed the experiment; ZL performed the majority of the experiments; JW, NZ, JY, ZW, and JM helped with the experiments; HW, YY, and KQ helped analyze the experimental results; ZL and YC wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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