

JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Suppression of the TLR3 mediated pro-inflammatory gene expressions by PPDPF in chicken DF-1 cells
Running Title (within 10 words)	PPDFP as a TLR3 signaling suppressor in chicken cells
Author	Eunmi Hwang ^{1,†} , Hyungkuen Kim ^{1,†} , Anh Duc Truong ² , Sung-Jo Kim ^{1,*} , Ki-Duk Song ^{2,*}
Affiliation	¹ Division of Cosmetics and Biotechnology, College of Life and Health Sciences, Hoseo University, Baebang, Asan 31499, Korea ² ³ Department of Agricultural Convergence Technology, Jeonbuk National University, Jeonju 54896, Korea
ORCID (for more information, please visit https://orcid.org)	Eunmi Hwang (https://orcid.org/0000-0001-8166-384X) Hyungkuen Kim (https://orcid.org/0000-0001-7508-9933) Anh Duc Truong (https://orcid.org/0000-0002-2472-8165) Sung-Jo Kim (https://orcid.org/0000-0003-4590-3644) Ki-Duk Song (https://orcid.org/0000-0003-2827-0873)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ015612)" Rural Development Administration, Republic of Korea.
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Song KD, Kim SJ. Methodology: Hwang E, Kim H. Investigation: Hwang E, Kim H. Writing - original draft: Hwang E, Kim H. Writing - review & editing: Song KD, Kim SJ, Truong AD.
Ethics approval and consent to participate	Approved by the Ministry of Agriculture and Rural Development of Vietnam (TCVN 8402:2010 and TCVN 8400-26:2014).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Ki-Duk, Song
Email address – this is where your proofs will be sent	kiduk.song@jbnu.ac.kr
Secondary Email address	kiduk.song@gmail.com
Address	567 Baek-jedaero, Deokjin gu, Jeonju, Republic of Korea
Cell phone number	+82-10-5622-1158
Office phone number	+82-63-219-5523
Fax number	+82-63-270-4739

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Sung-Jo, Kim
Email address – this is where your proofs will be sent	sungjo@hoseo.edu
Secondary Email address	sungjo@gmail.com
Address	Hoseo University, Baebang, Asan 31499, Korea
Cell phone number	+82-10-7268-9981
Office phone number	+82-41-540-5571
Fax number	+82 41-540-9538

Suppression of the TLR3 mediated pro-inflammatory gene expressions by PPDPF in chicken DF-1 cells

Eunmi Hwang^{1,†}, Hyungkuen Kim^{1,†}, Anh Duc Truong², Sung-Jo Kim^{1,*}, Ki-Duk Song^{3,4*}

† These authors contributed equally to this work

* Corresponding authors

¹ Division of Cosmetics and Biotechnology, College of Life and Health Sciences, Hoseo University, Baebang, Asan 31499, Korea

² Department of Biochemistry and Immunology, Vietnam National Institute of Veterinary Research, 86 Truong Chinh, Dong Da, Ha Noi, 80000, Vietnam

³ Department of Agricultural Convergence Technology, Jeonbuk National University, Jeonju 54896, Korea

⁴ The Animal Molecular Genetics and Breeding Center, Jeonbuk National University, Jeonju 54896, Korea

ORCID

Eunmi Hwang

<https://orcid.org/0000-0001-8166-384X>

Hyungkuen Kim

<https://orcid.org/0000-0001-7508-9933>

Anh Duc Truong

<https://orcid.org/0000-0002-2472-8165>

Sung-Jo Kim

<https://orcid.org/0000-0003-4590-3644>

Ki-Duk Song

<https://orcid.org/0000-0003-2827-0873>

Abstract

Toll-like receptors (TLRs), as a part of innate immunity, plays an important role in detecting pathogenic molecular patterns (PAMPs) which are structural components or product of pathogens and initiate host defense systems or innate immunity. Precise negative feedback regulations of TLR signaling are important in maintaining homeostasis to prevent tissue damage by uncontrolled inflammation during innate immune responses. In this study, we identified and characterized the function of the pancreatic progenitor cell differentiation and proliferation factor (PPDPF) as a negative regulator for TLR signal-mediated inflammation in chicken.

Bioinformatics analysis showed that the structure of chicken PPDPF evolutionarily conserved amino acid sequences with domains, i.e., SH3 binding sites and CDC-like kinase 2 (CLK2) binding sites, suggesting that relevant signaling pathways might contribute to suppression of inflammation. Our results showed that stimulation with polyinosinic:polycytidylic acids (Poly(I:C)), a synthetic agonist for TLR3 signaling, increased the mRNA expression of PPDPF in chicken fibroblasts DF-1 but not in chicken macrophage-like cells HD11. In addition, the expression of pro-inflammatory genes stimulated by Poly(I:C) were reduced in DF-1 cells which overexpress PPDPF. Future studies warrant to reveal the molecular mechanisms responsible for the anti-inflammatory capacity of PPDPF in chicken as well as a potential target for controlling viral resistance.

Keywords: chicken, DF-1, innate immunity, inflammation, Toll-like receptor 3, pancreatic progenitor cell differentiation and proliferation factor

Introduction

Innate immunity, known as a host defense system, recognizes and eliminates pathogens. Toll-like receptors (TLRs) play a key role in detecting pathogens, such as bacteria, viruses, and chemicals as well as activating innate immunity [1, 2]. TLR3 activates the inflammatory pathway by recognizing double-stranded RNA produced by viral infection. [3, 4]. Inflammation maintains homeostasis and prevents further infection, but excessive inflammation also damages normal cells [5]. TLR3 activation by highly pathogenic avian influenza A H5N1 virus, pandemic H1N1 virus, and dsRNA analog polyinosinic:polycytidylic acids (Poly(I:C)) induces inflammation and impairment of lung function, and knockout of TLR3 was positive for improvement of lung innate immunity and survival rate [6, 7]. Therefore, TLR3 inhibition is a good target for improving the survival rate of chickens against respiratory infectious dsRNA virus disease. Functional feed additives such as alpha-lipoic acid (ALA) inhibit TLR3 activity and improve chicken meat quality and productivity, but are not practical due to their high cost [8, 9]. In chickens, it is an attractive goal to genetically understand the mechanisms of TLR3 regulation and increase its regulatory efficiency. TLR regulatory mechanisms are well established in humans, but do not apply equally in chickens. In the case of human TLR4, MD-2 protein acts as an activity inhibitor of TLR4, whereas chicken TLR4 requires complex formation with MD-2 protein for pathogen detection [10]. In the case of TLR3, the amino acid sequence of the intracellular region, Toll/interleukin-1 receptor (TIR), is markedly different in birds and primates (Figure 1). The regulatory mechanisms of chicken TLR3 signaling remain to be studied.

In this study, we identified the pancreatic progenitor cell differentiation, and proliferation factor (PPDPF) as a potential inflammatory regulator in the chicken. PPPDF is a key modulator of exocrine pancreatic development and transcriptional target of retinoic acid (RA) and pancreas transcription factor 1a (PTF1A) [11, 12]. In zebrafish, PPDPF has been shown to promote

pancreatic exocrine gland growth and differentiation while inhibiting pancreatic endocrine gland growth and secretion. PPDPF gene is highly overexpressed in ovarian cancers and upregulated DNA replication pathway [13]. Furthermore, the PPDPF gene was discovered in prostate tumors as a genomic marker that can be utilized to predict biochemical recurrence [14]. Liver-specific PPDPF overexpression effectively inhibits HFD-induced mTOR signaling activation and hepatic steatosis in mice [15]. We investigated the levels of *PPDPF* expression in chicken organs and TLR3-activated chicken cells, as well as the role of PPDPF in TLR3 expression and activation in chicken cells. We also explain the diversity of PPDPF across the species and suggest chicken PPDPF as a factor for TLR3 activation and inflammation.

Materials and Methods

Bioinformatics of PPDPF

PPDPF protein amino acids sequences, i.e., chicken (*Gallus gallus*; XP_027329124.1), duck (*Anas platyrhynchos*); XP_027329124.1), human (*Homo sapiens*; Q9H3Y8.1), porcine (*Sus scrofa*; XP_020933363.1), bovine (*Bos Taurus*; NP_001068762.1), equine (*Equus caballus*; XP_023482697.1), murine (*Mus musculus*; Q9CR37), and feline (*Felis catus*; XP_003983360)

were retrieved from National Center for Biotechnology Information, U.S. National Library of Medicine, USA. Predicted phosphorylation site was evaluated using NetPhos-3.1 service (DTU Health Tech, Lyngby, Denmark) [16]. Predicted ubiquitination site was evaluated using RUBI Version 1.0 (BioComputing UP, Padua, Italy) [17] or obtained from neXtProt database (Swiss Institute of Bioinformatics, Lausanne, Switzerland). The structure of chicken PPDPF was predicted using IntFOLD server [18, 19]. 3-dimensional (3D) structure was constructed by using PyMol software (Schrödinger, NY, USA).

Cell culture condition

DF-1 chicken embryonic fibroblast cell line (Cat# CRL-12203, ATCC, Manassas, VA, USA) and the HD-11 chicken macrophage-like cell line [20] were cultured with Dulbecco's modified Eagle's medium (DMEM; Cat# 10-013-CVR, Corning, Corning, NY, USA) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) in a 5% CO₂ atmosphere at 37°C. The HD-11 cell line was provided by Dr. Hyun S. Lillehoj at the Agricultural Research Services (ARS) at the United States Department of Agriculture (USDA), Beltsville, Maryland, USA.

Chicken tissue collection

The specific-pathogen-free White Leghorn chickens (4-weeks old) were purchased from the Poultry Research Centre of the National Institute of Animal Science (NIAS; Hanoi, Vietnam). The chickens were given unlimited access to antibiotic-free feed and water. A total of 5 tissue samples were collected from the chickens, and placed in liquid nitrogen for total RNA extraction. All the experiments were conducted in compliance with the institutional rules for the care and use of laboratory animals, as well as implementing the protocol approved by the Ministry of Agriculture and Rural Development of Vietnam (TCVN 8402:2010 and TCVN 8400-26:2014).

RNA isolation and cDNA synthesis

Total RNA was isolated from the DF-1 cells, HD-11 cells, and lung tissue of White Leghorn chickens using a TRIzol reagent (Cat# 15596018, Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using WizScript cDNA Synthesis Kit (Cat# W2202, Wizbiosolutions, Seongnam, Korea).

Real-time PCR (RT-PCR)

Real-time PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 10 ng cDNA, SYBR Green qPCR Master Mix (Cat# DQ385-40h, Biofact, Daejeon, Korea), and 1 pM primer (Table 2). Levels of *TLR3*, *TNF receptor-associated factor 3 (TRAF3)*, *Toll-Like Receptor Adaptor Molecule 1 (TICAM1)*, *nuclear factor- κ B1 (NF- κ B1)*, *tumor necrosis factor α (TNF α)*, and *cyclooxygenase2 (COX2)* mRNA were measured [21]. mRNA fold-change was normalized to β -actin mRNA using $2^{-\Delta\Delta C_t}$ method [22].

Cloning of chicken *PPDPF*

cDNA from lung tissue of White Leghorn chickens was used for *PPDPF* cloning. PCR was performed for isolation and cloning of *PPDPF* using *Pfu* DNA polymerase with specific primers (Table 1) under the following conditions: 95°C for 5 min, 30 cycles of 95°C 30 sec, 56°C 45 sec, 72°C 50 sec, and the final extension for 10 min at 72°C. Newly synthesized DNA fragments were purified using Gel Purification kit (Cat# K-303501, Bioneer, Daejeon, Korea). DNA fragments and pEGFP-N3 vectors were digested using *HindIII* and *BamHI* restriction enzymes. DNA fragment was inserted into the vector using the TOPcloner kit (Cat# EZ002S, Enzymomics, Daejeon, Korea) and transformed into *E. coli* DH5 α . The plasmid was purified using the Plasmid Mini Extraction Kit (Cat# K-3030, Bioneer, Daejeon, Korea) and clones containing *PPDPF* CDS were selected through sequencing (Bioneer). All processes were carried out according to the manufacturer's instructions.

Transfection of *PPDPF*

Chicken DF-1 cells were transfected with pEGFP-N3 vectors concentration at $1 \mu\text{g} / 1.0 \times 10^6$ cells using Lipofectamine 3000 transfection reagent (Cat# L3000008, Invitrogen) according to the manufacturer's instructions.

Fluorescence microscopy

Cells were washed with phosphate buffered saline (PBS; pH 7.4), fixed in 3.8% formaldehyde for 15 min, and stained with 4',6-diamidino-2-phenylindole (DAPI, $1 \mu\text{g}/\text{mL}$). Fluorescence was visualized using a DMi8 fluorescence microscope (Leica, Deerfield, IL, USA) and LAS X program (Leica). Lightness and contrast of images were processed using Photoshop CC program (Adobe Systems, San Jose, CA, USA).

Cell viability assay

Cells were cultured in 96-well plates with 10% (v/v) WST-1 reagent (Cat# EZ-3000, DoGenBio, Seoul, Korea) for 2 h and measured the absorbance at 450 nm using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland).

Statistical Analysis

Results were obtained from three separate experiments ($n = 3$) and analyzed using GraphPad PRISM 8 software (GraphPad Software, San Diego, CA, USA) and Microsoft Excel software (Microsoft, Redmond, WA, USA). The data are expressed as mean \pm standard deviation (SD). The p -value was calculated using an analysis method suitable for each experiment and specified in figure legends. Results were considered statistically significant when the p -value was < 0.05 .

Results

The intracellular domain TIR regulates TLR3 activity through adapter protein binding and signaling when TLR3 is activated by ligand [23]. Therefore, we identified the possibility at the amino acid sequence level for differences in the regulation of TLR3 signaling in primates and avian. Amino acid sequences of the TIR domain between two avian species, chickens (*Gallus gallus*, ADZ48550.1) and ducks (*Anas platyrhynchos*, AIW60885.1) were very similar. TIR domain sequence homology was also observed between two primates, i.e., human (*Homo sapiens*, AAH96335.1) and chimpanzees (*Pan troglodytes*, BAG55029.1). Comparison of the TIR domain sequences between avian and primates revealed very low homology (Figure 1), suggesting the possibility that TLR3 signaling of avian species may be distinct from that of primates.

Bioinformatics analysis of chicken PPDPF protein

We performed an amino acid sequence-based analysis to analyze the interspecies diversity of PPDPF proteins and predict their functions. The PPDPF protein sequences were highly conserved up to the 76th amino acid (AA). The predicted ubiquitination site (99Lys) was identified only in human, bovine, equine, murine, and feline sequences (Figure 2A). The homology of the PPDPF protein sequence to chicken PPDPF averaged only 50% in the species we investigated (Figure 2A and 2B). Amino acid sequence diversity dropped sharply at 77th AA across all species investigated in this study (Figure 2A and 2C). To discover the function of chicken PPDPF, the 3D structure of the chicken PPDPF protein (NP_001183966.1) was predicted using the IntFOLD server. In our protein model, chicken PPDPF was identified as a structure with one alpha-helix (Figure 2D). In the conserved region of PPDPF (1-76AA), phosphorylation sites are located. And the CDC like kinase (CLK) 2 kinase sites, a SH3 domain binding site, and ATP/GTP binding sites reported in PPDPF were located [11]. In the variable

region (77-113AA), the alpha helix structure and the predicted ubiquitination site were located (Figure 2D). These results suggest the possibility that PPDPF may play a role that differs from species to species.

***PPDPF* mRNA expression induced by Poly(I:C) treatment in chicken cells**

To confirm whether the expression of *PPDPF* in chickens is affected by dsRNA, *PPDPF* mRNA fold-change was measured by RT-PCR in Poly(I:C)-treated chicken cell lines. In DF-1 cells, a chicken embryonic fibroblast cell line, *PPDPF* mRNA expression was increased 25-fold by treatment with 10 µg/mL Poly(I:C) (Figure 3A). And, as expected, in chicken macrophage-like cell line HD-11 cells lacking exogenous dsRNA detection ability [24], Poly(I:C) treatment did not affect the expression of *PPDPF* (Figure 3B). To evaluate the *PPDPF* expression levels for each tissue, RT-PCR was performed with cDNA from chicken organs. The expression levels of *PPDPF* were the highest in the lungs and the lowest in the heart among the organs which we investigated (Figure 3C). Through these results, we confirmed the possibility that *PPDPF* expression was induced by Poly(I:C), a TLR3 activator, in chickens and is related to the immune response in the lungs.

Effects of *PPDPF* on the inflammation in DF-1 cells

To determine the effect of *PPDPF* expression on TLR3 inflammatory response in chicken cells, we constructed a chicken *PPDPF* expression vector based on the coding sequences (CDS) of *PPDPF* mRNA (accession no. NM 001197037.1) (Figure 4A). PCR was used to isolate CDS from chicken *PPDPF*, which was then introduced into the pEGFP-N3 vector (Figure 4B) (Figure 4C). Transfection of *PPDPF* inserted pEGFP-N3 vector into DF-1 cells elevated *PPDPF* mRNA expression, as validated by RT-PCR (Figure 4D). DF-1 cells were cultured with Poly(I:C) for 24 hours to examine if *PPDPF* expression alters the expression of pro-inflammatory genes produced

by Poly(I:C), and fluorescence microscopy and RT-PCR were performed (Figure 5A). The PPDPF protein was uniformly located in the cell including the nucleus. TLR3 signaling genes (*TLR3*, *TRAF3*, and *TICAM1*), as well as pro-inflammatory genes (*NF-B1* and *TNF- α*), were increased by Poly(I:C) treatment, however PPDPF overexpression suppressed *TLR3*, *TRAF3*, *TICAM1*, and *TNF- α* expression (Figure 5C). It is of note that PPDPF transfection did not induce toxicity in DF-1 cells (Figure 5D). These results suggest that overexpression of PPDPF in chicken fibroblasts is a potential TLR3 inhibitor to downregulate the mRNA expression of TLR3-related genes induced by Poly(I:C). Through the above results, we confirmed that PPDPF inhibits Poly(I:C)-induced TLR3 mRNA expression in DF-1 cells and might act as a negative regulator for dsRNA-induced inflammation.

Discussion

In chickens, TLR3 is important for immunity against major infectious diseases that threaten the avian industry, such as avian influenza virus (AIV) and Newcastle disease virus (NDV) [25, 26]. But excessive inflammation might be responsible for reduced chicken productivity [27, 28]. TLR-mediated inflammation against invading pathogens should be dampened to maintain homeostasis to prevent the potential damage resulted from uncontrolled responses. Numerous negative regulatory molecules have been identified and characterized at multiple levels [29]. However, no negative regulators for TLR have been reported in livestock, including poultry. In this study, we discovered PPDPF as a protein capable of suppressing TLR3-mediated inflammatory responses in DF-1 cells. The main functions of PPDPF in mammals were well reported as a key regulator of development of pancreas in human. In contrast, the structure and function of PPDPF have not yet been reported in chickens.

In this study, we identified the potential for differences in TLR3 signaling and binding proteins in humans and chickens based on the amino acid sequence of TIR domain (Figure 1). And we predicted the function and structure of PPDPF through bioinformatics (Figure 2). The evolutionarily conserved domains present in chicken PPDPF proteins, the CLK2 kinase binding site, SH3 domain binding site, and ATP/GTP binding site, reveal the potential of PPDPF to participate in signaling pathways or act as enzymes. (Fig. 2A). The presence of SH3 domains in proteins suggests potential involvement in various signaling pathways by protein tyrosine kinases through protein-protein interactions [30]. CLK2 protein is implicated in a variety of signaling pathways, including regulation of inflammation and viral resistance [31, 32], and cell proliferation [33]. Presence of ATP/GTP binding site in protein, it can use the energy of ATP for enzymatic action [34]. The role of the chicken PPDPF domain should be verified through a loss of function study.

We examined the relationship between TLR3 signaling and PPDPF in chicken cells. The expression level of PPDPF is upregulated by TLR3 stimulation with Poly(I:C) in DF-1 cells but not in HD-11 cells (Figure 3A and 3B). HD11 cells have low reactivity to exogenous dsRNA, and TLR3 activation is completed in a very short time, so PPDPF expression may not be induced even with poly(I:C) treatment [24, 35]. TLR3 is mainly located on the cell surface of non-immune cells, including fibroblasts [36]. The DF-1 chicken embryo fibroblast cell line is derived from chicken embryos and has active TLR3 signaling pathways [37, 38], demonstrating that DF-1 cells might be a suitable model to study the TLR3 responses in chickens.

It is of note that, in Poly(I:C) treated DF-1 fibroblasts, PPDPF-eGFP is mainly located in the nucleus (see Figure 5B), and mechanism behind this localization remains to be studied, but at least, it seems that PPDPF may not have direct interaction with TLR3. Mechanism behind the localization of PPDPF remains to be studied. Overexpression of PPDPF reduced pro-inflammatory gene expression in DF-1 chicken embryonic fibroblasts. These results indicate the possibility of

PPDPF acting as a negative regulator of TLR3 mediated inflammation in DF-1 chicken embryonic fibroblasts. NF- κ B1 can induce systemic pro-inflammatory gene expression under stress conditions, so inhibition of *NF- κ B1* expression by PPDPF could be effective in suppressing inflammation in poultry [39].

Future studies require evaluation of the effect of PPDPF protein on TLR3 signaling, inflammatory response, and binding protein through loss of function study of chicken PPDPF protein. Nevertheless, based on the sequence and predicted structure of the PPDPF protein and the study in PPDPF-overexpressing DF-1 cells, we suggest the possibility that PPDPF may participate in TLR3-mediated inflammatory responses in chickens.

Acknowledgement

This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ015612)" Rural Development Administration, Republic of Korea.

References

1. Bsibsi M, Ravid R, Gveric D, van Noort JM. Broad expression of Toll-like receptors in the human central nervous system. *J Neuropathol Exp Neurol*. 2002;61(11):1013-21.
2. Yao C, Oh JH, Lee DH, Bae JS, Jin CL, Park CH, et al. Toll-like receptor family members in skin fibroblasts are functional and have a higher expression compared to skin keratinocytes. *Int J Mol Med*. 2015;35(5):1443-50.
3. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol*. 2004;4(7):499-511.
4. Kariko K, Buckstein M, Ni H, Weissman D. Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity*. 2005;23(2):165-75.
5. Chovatiya R, Medzhitov R. Stress, inflammation, and defense of homeostasis. *Mol Cell*. 2014;54(2):281-8.
6. Leung YC, Nicholls JM, Ho CK, Sia SF, Mok CK, Valkenburg SA, et al. Highly pathogenic avian influenza A H5N1 and pandemic H1N1 virus infections have different phenotypes in Toll-like receptor 3 knockout mice. *The Journal of general virology*. 2014;95(Pt 9):1870.
7. Stowell NC, Seideman J, Raymond HA, Smalley KA, Lamb RJ, Egenolf DD, et al. Long-term activation of TLR3 by poly (I: C) induces inflammation and impairs lung function in mice. *Respiratory research*. 2009;10(1):1-14.
8. Mora Izaguirre O, Quester K, Petranovski V, González Dávalos L, Piña Garza E, Shimada Miyasaka A, et al. Effect of nanostructured zeolite with lipoic acid on performance and carcass yield in broiler. *Revista mexicana de ciencias pecuarias*. 2018;9(2):185-202.
9. Guo Z, Li J, Zhang L, Jiang Y, Gao F, Zhou G. Effects of alpha-lipoic acid supplementation in different stages on growth performance, antioxidant capacity and meat quality in broiler chickens. *British poultry science*. 2014;55(5):635-43.
10. Keestra AM, van Putten JP. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. *J Immunol*. 2008;181(6):4354-62.
11. Jiang Z, Song JB, Qi F, Xiao A, An XZ, Liu NA, et al. Exdpf Is a Key Regulator of Exocrine Pancreas Development Controlled by Retinoic Acid and ptf1a in Zebrafish. *Plos Biol*. 2008;6(11):2450-64.
12. Breunig M, Hohwieler M, Seufferlein T, Glöckner J, Liebau S, Kleger AJZfG. PDPF impacts pancreatic progenitor cell formation derived from human pluripotent stem cell. *J Zeitschrift für Gastroenterologie*. 2018;56(08):KV 101.
13. Xiao Y, Lai Y, Yu Y, Jiang P, Li Y, Wang C, Zhang R. The Exocrine Differentiation and Proliferation Factor (EXDPF) Gene Promotes Ovarian Cancer Tumorigenesis by Up-Regulating DNA Replication Pathway. *Front Oncol*. 2021 May 10;11:669603. doi:

10.3389/fonc.2021.669603. PMID: 34041032; PMCID: PMC8141798

14. Vittrant B, Leclercq M, Martin-Magniette ML, Collins C, Bergeron A, Fradet Y, Droit A. Identification of a Transcriptomic Prognostic Signature by Machine Learning Using a Combination of Small Cohorts of Prostate Cancer. *Front Genet.* 2020 Nov 25;11:550894. doi: 10.3389/fgene.2020.550894. PMID: 33324443; PMCID: PMC7723980).
15. Ma N, Wang YK, Xu S, Ni QZ, Zheng QW, Zhu B, Cao HJ, Jiang H, Zhang FK, Yuan YM, Zhang EB, Chen TW, Xia J, Ding XF, Chen ZH, Zhang XP, Wang K, Cheng SQ, Qiu L, Li ZG, Yu YC, Wang XF, Zhou B, Li JJ, Xie D. PDPF alleviates hepatic steatosis through inhibition of mTOR signaling. *Nat Commun.* 2021 May 24;12(1):3059. doi: 10.1038/s41467-021-23285-8. PMID: 34031390; PMCID: PMC8144412.
16. Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol.* 1999;294(5):1351-62.
17. Walsh I, Di Domenico T, Tosatto SC. RUBI: rapid proteomic-scale prediction of lysine ubiquitination and factors influencing predictor performance. *Amino Acids.* 2014;46(4):853-62.
18. McGuffin LJ, Adiyaman R, Maghrabi AHA, Shuid AN, Brackenridge DA, Nealon JO, et al. IntFOLD: an integrated web resource for high performance protein structure and function prediction. *Nucleic Acids Res.* 2019;47(W1):W408-W13.
19. McGuffin LJ, Shuid AN, Kempster R, Maghrabi AHA, Nealon JO, Salehe BR, et al. Accurate template-based modeling in CASP12 using the IntFOLD4-TS, ModFOLD6, and ReFOLD methods. *Proteins.* 2018;86:335-44.
20. Beug H, von Kirchbach A, Doderlein G, Conscience JF, Graf T. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell.* 1979;18(2):375-90.
21. Matsumoto M, Oshiumi H, Seya T. Antiviral responses induced by the TLR3 pathway. *Rev Med Virol.* 2011;21(2):67-77.
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods.* 2001;25(4):402-8.
23. Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *Int Immunol.* 2004;16(8):1143-54.
24. Karpala AJ, Lowenthal JW, Bean AGJD, Immunology C. Activation of the TLR3 pathway regulates IFN β production in chickens. 2008;32(4):435-44.
25. Cheng J, Sun Y, Zhang X, Zhang F, Zhang S, Yu S, et al. Toll-like receptor 3 inhibits Newcastle disease virus replication through activation of pro-inflammatory cytokines and the type-1 interferon pathway. 2014;159(11):2937-48.

26. Wang Q, Miller DJ, Bowman ER, Nagarkar DR, Schneider D, Zhao Y, et al. MDA5 and TLR3 initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness. 2011;7(5):e1002070.
27. Nailwal H, Chan FK-MJCD, Differentiation. Necroptosis in anti-viral inflammation. 2019;26(1):4-13.
28. Jiang Z, Schatzmayr G, Mohnl M, Applegate TJPs. Net effect of an acute phase response—partial alleviation with probiotic supplementation. 2010;89(1):28-33.
29. Anwar MA, Basith S, Choi S. Negative regulatory approaches to the attenuation of Toll-like receptor signaling. *Experimental & molecular medicine*. 2013;45(2):e11-e.
30. Kurochkina N, Guha U. SH3 domains: modules of protein–protein interactions. *Biophysical reviews*. 2013;5(1):29-39.
31. Deshmukh V, O'Green AL, Bossard C, Seo T, Lamangan L, Ibanez M, et al. Modulation of the Wnt pathway through inhibition of CLK2 and DYRK1A by lorecivivint as a novel, potentially disease-modifying approach for knee osteoarthritis treatment. *Osteoarthr Cartilage*. 2019;27(9):1347-60.
32. Wong R, Balachandran A, Mao AY, Dobson W, Gray-Owen S, Cochrane A. Differential effect of CLK SR Kinases on HIV-1 gene expression: potential novel targets for therapy. *Retrovirology*. 2011;8(1):47.
33. Petsalaki E, Zachos G. Clks 1, 2 and 4 prevent chromatin breakage by regulating the Aurora B-dependent abscission checkpoint. *Nat Commun*. 2016;7:11451.
34. Chauhan JS, Mishra NK, Raghava GP. Identification of ATP binding residues of a protein from its primary sequence. *BMC Bioinformatics*. 2009;10:434.
35. Peroval MY, Boyd AC, Young JR, Smith AL. A critical role for MAPK signalling pathways in the transcriptional regulation of toll like receptors. *PLoS One*. 2013;8(2):e51243.
36. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol*. 2003;171(6):3154-62.
37. Tian W, Zhao C, Hu Q, Sun J, Peng XJD, Immunology C. Roles of Toll-like receptors 2 and 6 in the inflammatory response to *Mycoplasma gallisepticum* infection in DF-1 cells and in chicken embryos. 2016;59:39-47.
38. Foster DJV. Development of a spontaneously immortalized chicken embryo fibroblastic cell line. 1998;248(2):305-11.
39. Surai PF, Kochish, II, Kidd MT. Redox Homeostasis in Poultry: Regulatory Roles of NF-kappaB. *Antioxidants (Basel)*. 2021;10(2).

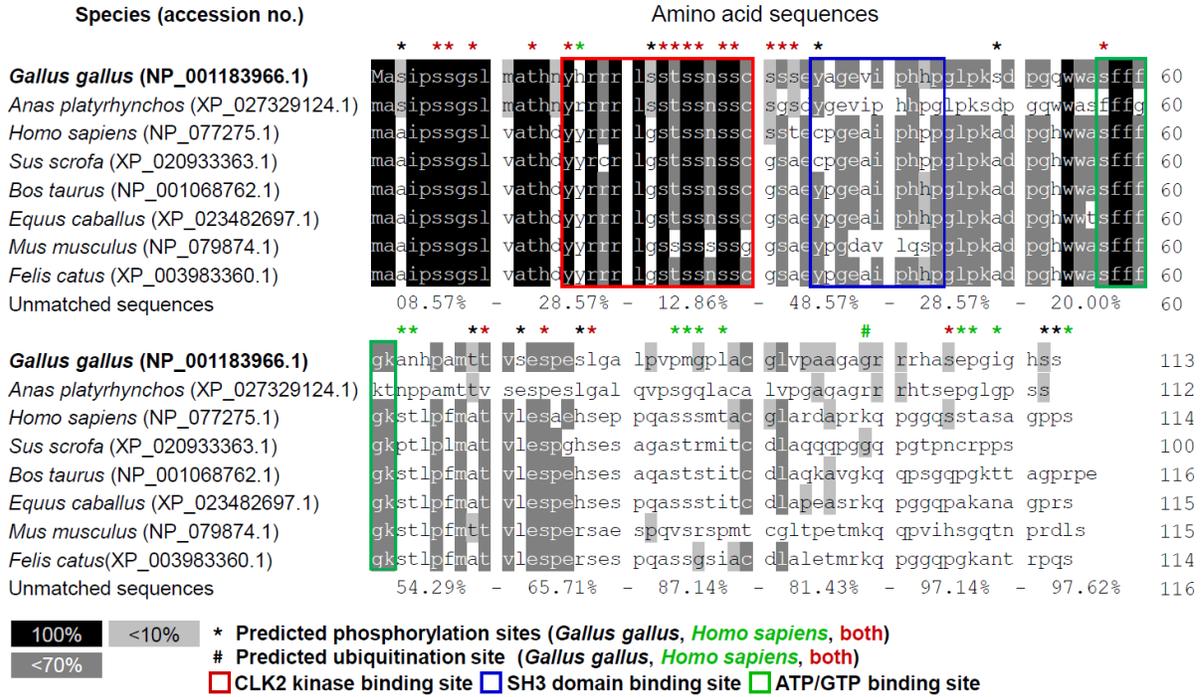
Figure Legends

Species (accession no.)	Amino acid sequences of TIR domain	
<i>Gallus gallus</i> (ADZ48550.1)	ydayvihark dtnwvltnft tleeneqfgv kfcleerdfe agisefeaii ncirrsrkii	60
<i>Anas platyrhynchos</i> (AIW60885.1)	ydayliharh drnwvlnkfi sleknkqfev rfcleerdfe agvsefeaii nsirrsrkii	60
<i>Homo sapiens</i> (AAH96335.1)	yiihaykdkd wwwehfssme kedqslkfcl eerdfeagvf eleaivnsik rsrkiifvit	60
<i>Pan troglodytes</i> (BAG55029.1)	yiihaykdkd wwwehfssme kedqslkfcl eerdfeagvf eleaivnsik rsrkiifvit	60
<i>Gallus gallus</i> (ADZ48550.1)	fiivtehlldg pwrkfkvnh alqqaieqsr dsiiilflhn iqdyk	105
<i>Anas platyrhynchos</i> (AIW60885.1)	fiivtehlldg pwrkfkvyh avgqaieqsr dsiiilvflhd iqdyklnhal clrrgmfrsr cilkwpaq	128
<i>Homo sapiens</i> (AAH96335.1)	hhllkdpick rfkvhhavqg aieqnl dsii lvfleecipdy k	101
<i>Pan troglodytes</i> (BAG55029.1)	hhllkdpvck rfkvhhavqg aieqnl dsii lvfleecipdy k	101

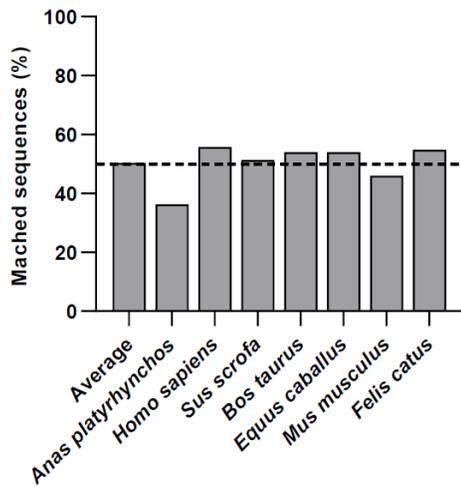
100%
≤50%

Figure 1. TIR amino acid sequences. Amino acid sequences of intracellular domain of TLR3 of chicken (*Gallus gallus*, ADZ48550.1), duck (*Anas platyrhynchos*, AIW60885.1), human (*Homo sapiens*, AAH96335.1), and chimpanzee (*Pan troglodytes*, BAG55029.1). Homology of amino acid sequences are highlighted as black (100%) or gray ($\leq 50\%$).

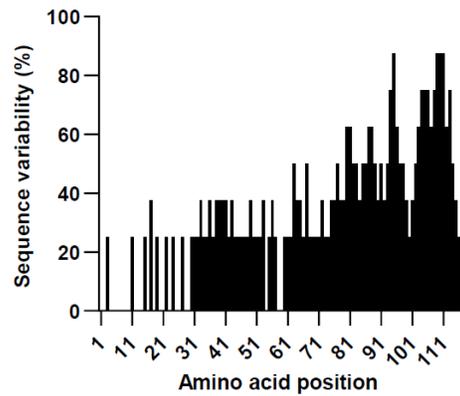
A



B



C



D

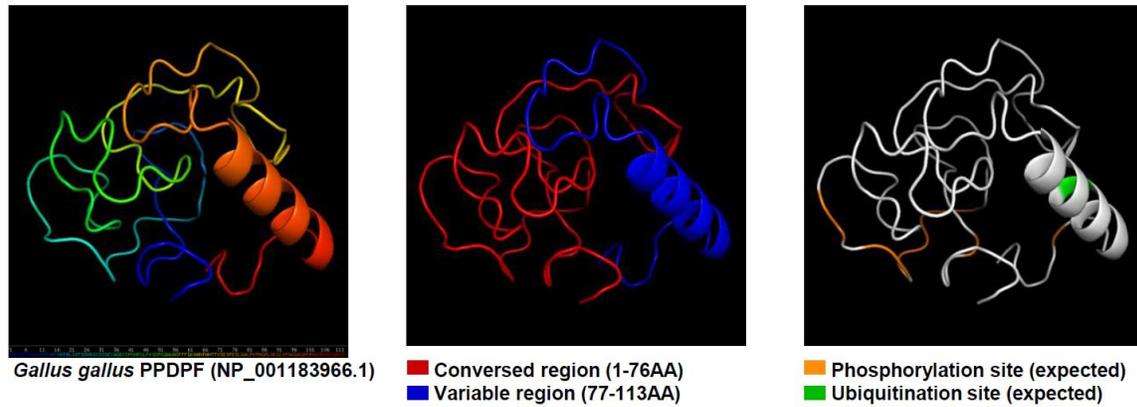


Figure 2. Bioinformatics analysis of chicken PPDPF proteins. (A) Amino acid sequences and motif of PPDPF from chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), human (*homo sapiens*), porcine (*Sus scrofa*), bovine (*Bos taurus*), equine (*Equus caballus*), murine (*Mus musculus*), and feline (*Felis catus*). (B) PPDPF amino acid sequence homology to chicken PPDPF protein. (C) Variability of amino acid sequences of PPDPF across the species. (D) Predicted protein structure of chicken (*Gallus gallus*) PPDPF.

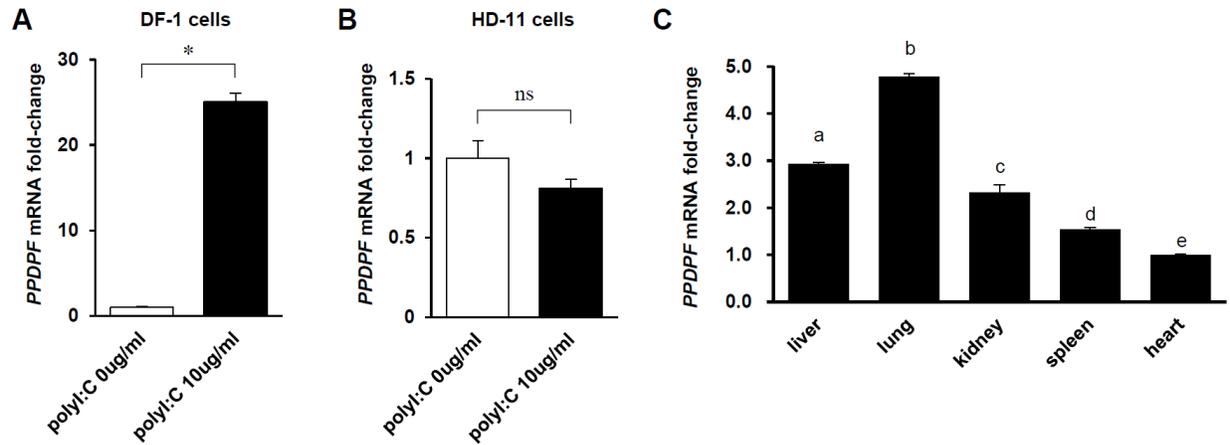


Figure 3. *PPDPF* mRNA expression induced by Poly(I:C) treatment in chicken cells. (A, B) *PPDPF* mRNA expression levels in chicken DF-1 fibroblasts (A) and HD-11 macrophage-like cells (B). mRNA fold-change was normalized to β -*actin* mRNA. Data are expressed as mean \pm SD (n = 3). *p < 0.01 and ^{ns}p > 0.05 by unpaired two-tailed Student's t-test. (C) mRNA expression level of *PPDPF* in liver, lung, kidney, spleen, and heart tissue of chicken. mRNA expression levels were measured by real-time PCR. mRNA fold-change was normalized to β -*actin* mRNA. Data are expressed as mean \pm SD (n = 3). Statistical significance was measured using one-way ANOVA.

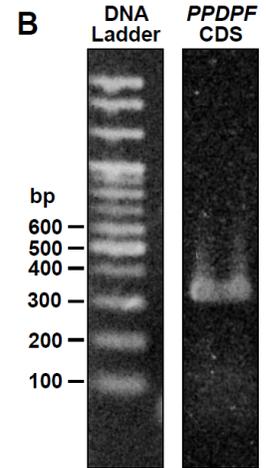
A

Gallus gallus PPDPF mRNA sequences (CDS)

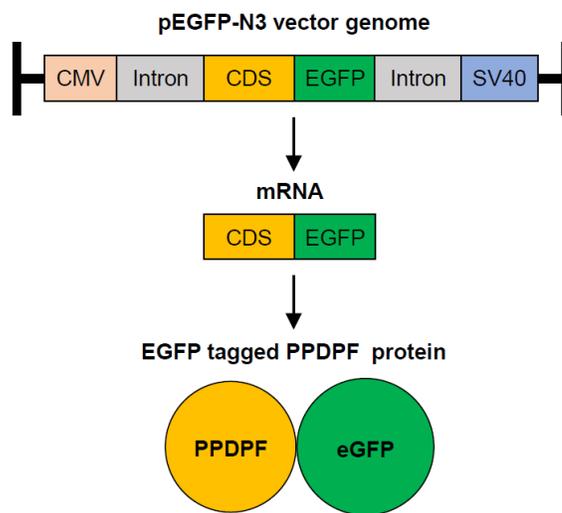
```

1 gcgcgcgggc tcaggccgtg cggagcggac gcgggtgctg cgctccctcg ggcgggggct
61 tcgagcgccc cgcggggccc cttaccctc cctttctct tccggacccc actgggcctt
121 agtgccggcc aggttttoca tcagcacaaa ccaggacccc ggagccatgg cctccatccc
181 atcgagcggc tcgcttatgg ccacgcacaa ctatcaccga aggcgtctga gctccacatc
241 cagcaacagc tctgcagta gctctgagta tgctggggag gtcatecccc accaccggg
301 tctgccc aaa tctgacccc gccagtggtg ggcagcttc ttctttggga aggccaatca
361 ccctgccatg acaactgtgt cagagtcccc agagagcttg ggagcgtgc cagtaccat
421 ggggcccctg gcgtgtgggc tggtgccagc agcaggagca ggacgcaggc gccatgccag
481 cgagccaggc attgggcact cctcctgagc tgcaatggcc cttcccttcc ctgcagagct
541 gcttgccagat gtatgtgctg ctttgggta agacaaggca gcagccccc cagcaattcc
601 tacaccagcc tcttactatt tctcaatgca accgtgcat ttctgtgcag attccgtaac
661 acgtttattg actttgtact tgtacaatgg ctactaataa actcgagcag ctttttagca
721 c

```



C



D

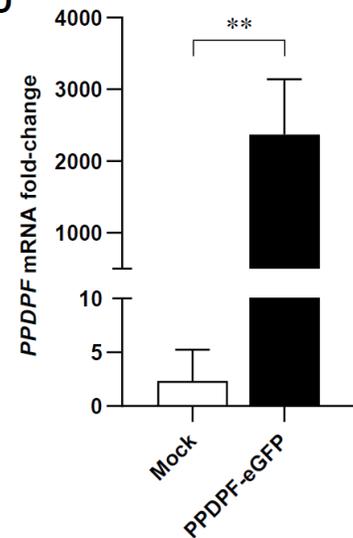


Figure 4. Construction of PPDPF-eGFP expression vector. (A) Reference sequences of *G. gallus* PPDPF mRNA (accession no. NM_001197037.1). (B) Full-length coding sequence (CDS) of chicken PPDPF was amplified using PCR and separated using agarose gel electrophoresis. (C) Design of pEGFP-N3 vector containing CDS of chicken PPDPF mRNA. (D) PPDPF mRNA expression levels in DF-1 cells were measured using RT-PCR after transfected for 48 h. mRNA fold-change was normalized to β -actin mRNA. Data are expressed as mean \pm SD (n = 3). **p < 0.01 by unpaired two-tailed Student's t-test.

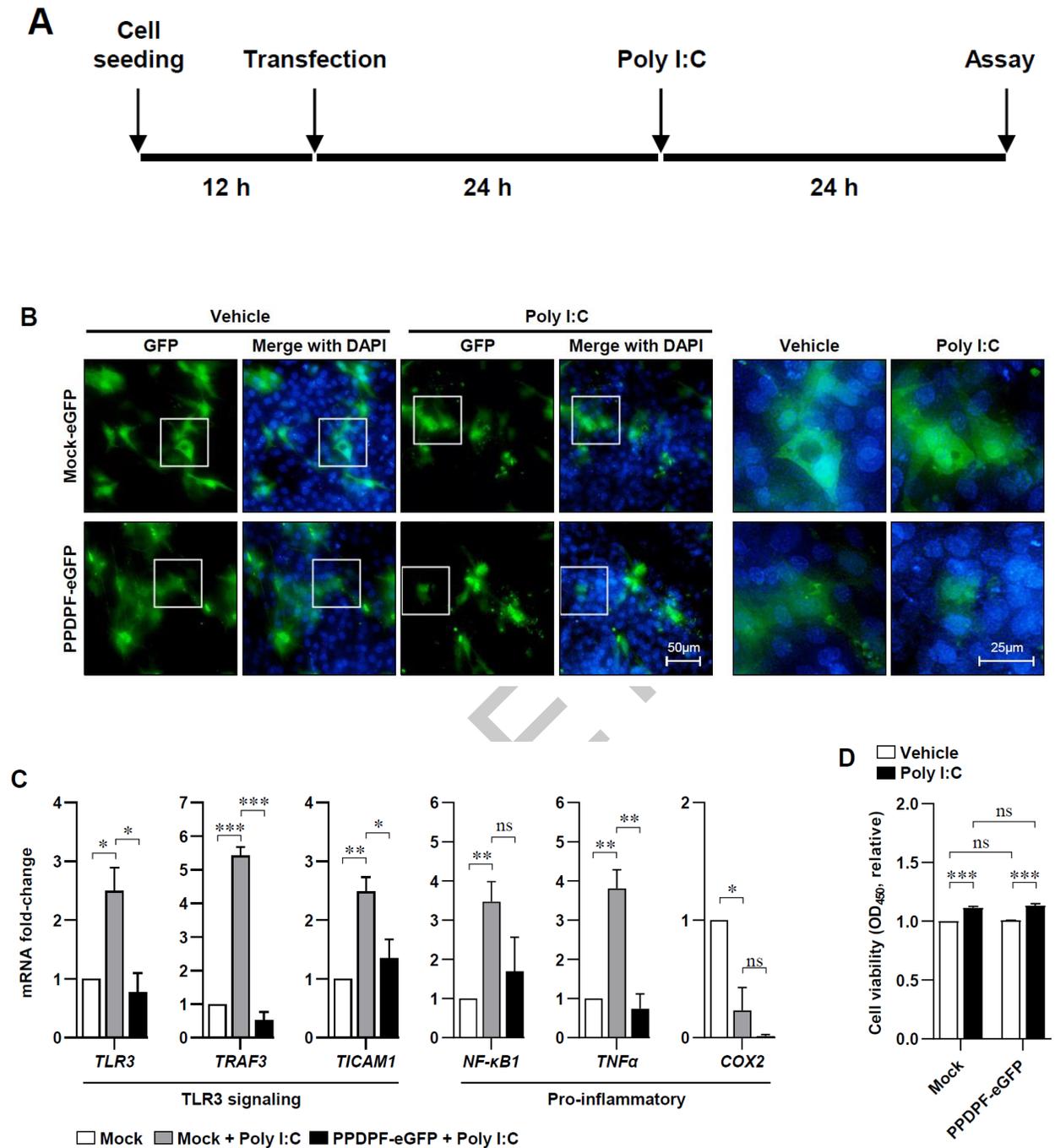


Figure 5. PDPDF suppresses pro-inflammatory gene expression in poly(I:C) treated DF-1 cells. (A) Experimental design. Poly(I:C) (10 μ g/mL) was treated 24 h after transfection. (B) Fluorescence microscopy of DF-1 cells transfected with pEGFP-N3 vector (scale bar = 50 μ m or 25 μ m). (C) mRNA fold-change of mRNA related with TLR3 response in DF-1 cells. mRNA fold-change was normalized to β -actin mRNA (D) Cell viability of DF-1 cells treated with

Poly(I:C) for 24 h. Data are expressed as mean \pm SD (n = 3). All data are expressed as mean \pm SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, and ^{ns}p > 0.05 by unpaired two-tailed Student's t-test.

ACCEPTED

Tables and Figures

Table 1. Primer Sequences for chicken *PPDPF* cloning. Primers for isolation of *PPDPF* were in outside of CDS. Primers for cloning containing *Hind*III (AAGCTT) or *Bam*HI (GGATCC) sequences. F: forward; R: reverse

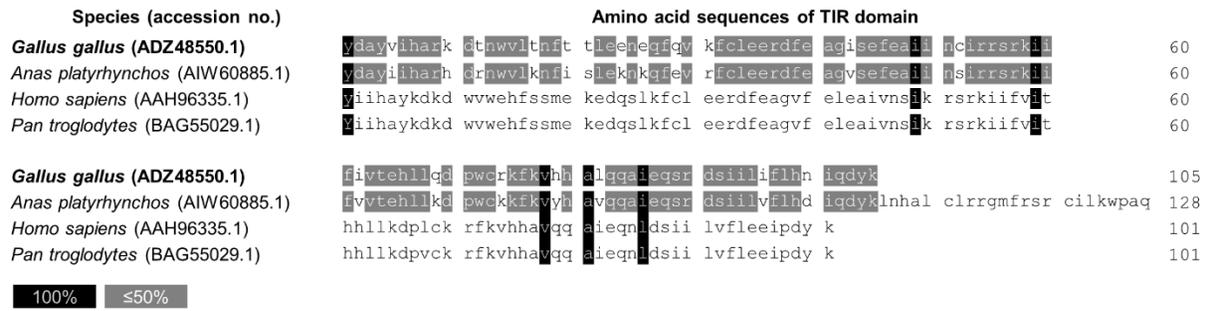
Purpose	Sequences (5' to 3')	Accession No.
Isolation	F: CCAGGTTTTCCATCAGCACAA R: AAGGGAAGGGCCATTGCAG	NM_001197037.1
Cloning	F: <u>AAGCTT</u> ATGGCCTCCATCCCATCGAGC R: <u>GGATCC</u> GGATGAGTGCCCAATGCCTGG	NM_001197037.1

ACCEPTED

Table 2. Primer sequences for DF-1 chicken embryonic fibroblasts. F: forward; R: reverse

Gene	Sequences (5' to 3')	Accession No.
<i>TLR3</i>	F: AAAGTGGGGCATTTCCTCCT R: TTGAGACCTTCGAATTCATGACC	NM_001011691.3
<i>NF-κB1</i>	F: TCTGGAGGTACAGTTCAAGAGC R: TGTGTTGTCCTCCTGGTATATCT	NM_205134.1
<i>TNFα</i>	F: GGTTCGAGTCGCTGTATCAGG R: ACTCCCACCACCCCAAATA	MF000729.1 MF801626.1
<i>COX2</i>	F: TAAAGCTTACGGCCATGGGG R: AGTGTCCTTCACTGTTGGTGG	NM_001167719.1 NM_001167718.1
<i>TRAF3</i>	F: TCAAGACCAGCTCTCAGCAG R: GAACTTTGCTAGTCTGCCCCG	XM_004936343.3 (Transcript variant X1~X8, X10~X12)
<i>TICAM1</i>	F: CACATCTGCTCAGGTGGGTC R: GGATGATGATGGAACGGGCA	NM_001081506.1
<i>β-Actin</i>	F: TGCTACGTCGCACTGGATTT R: AAAGATGGCTGGAAGAGGGC	NM_205518.1

Figure 1.



ACCEPTED

Figure 2.

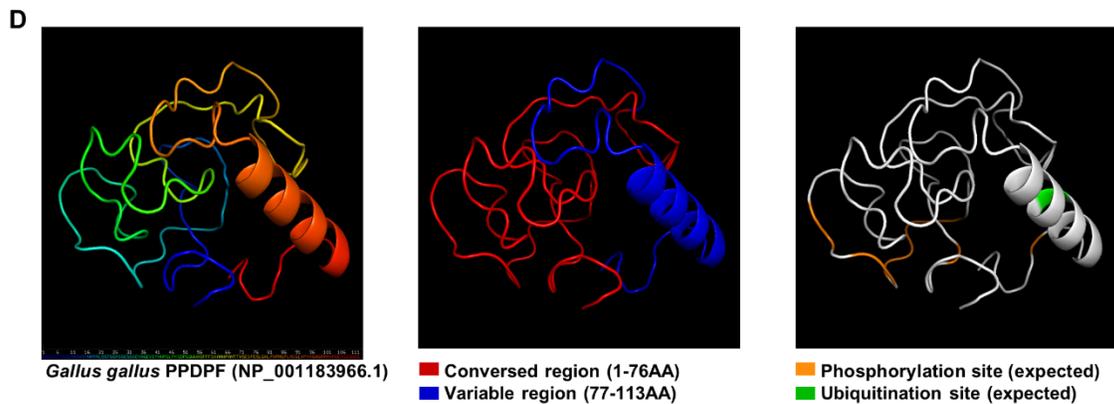
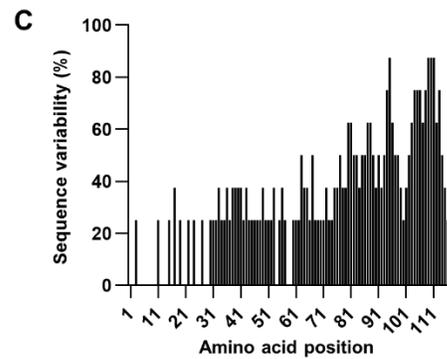
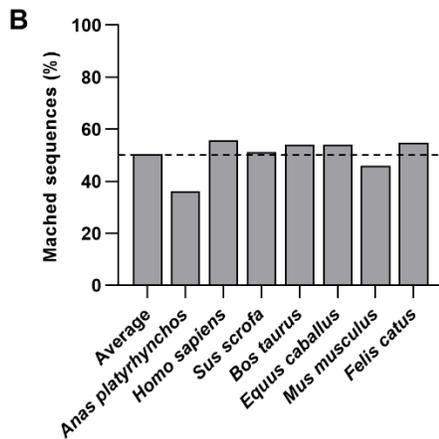
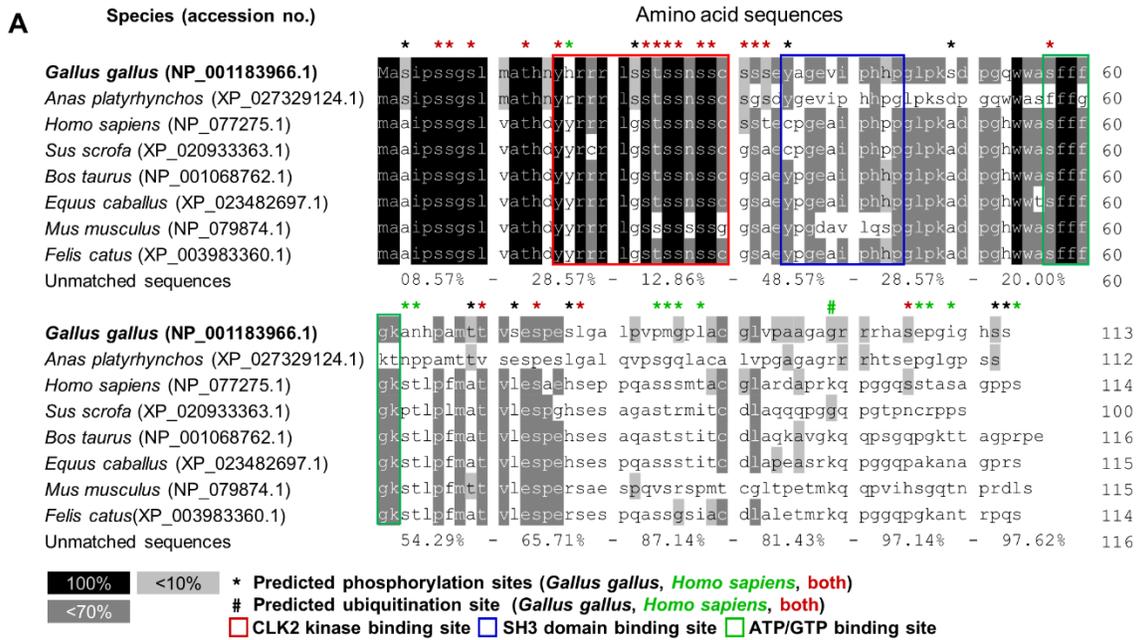
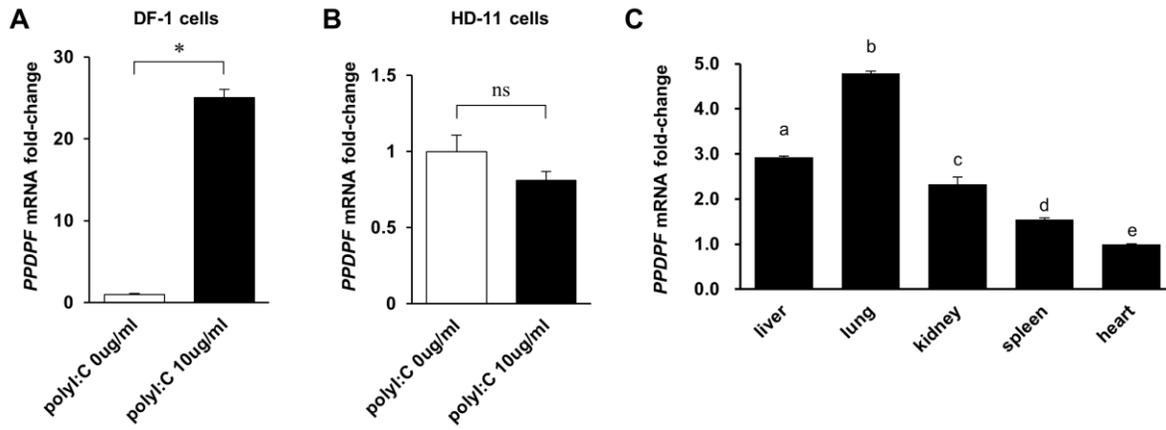


Figure 3.



ACCETEL

Figure 4.

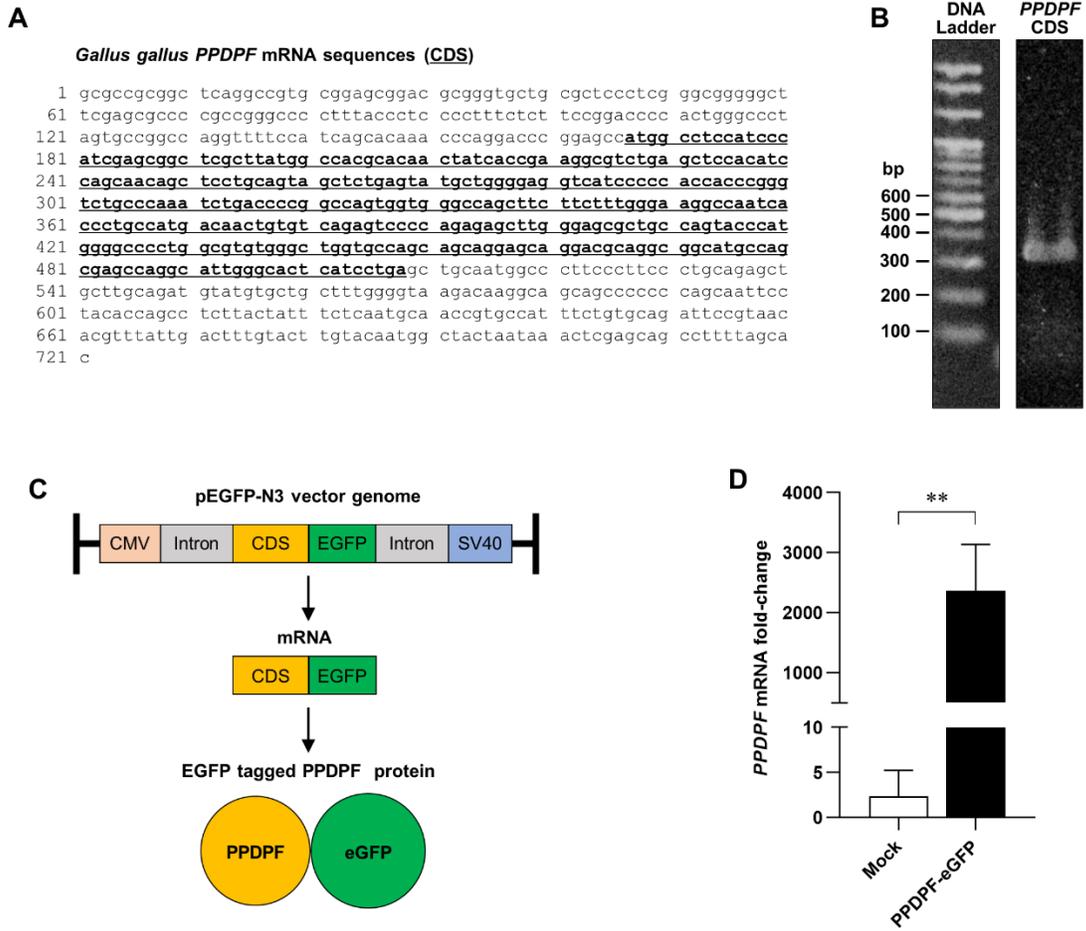


Figure 5.

