# Chicken miR-126-5p Negatively Regulates Antiviral Innate Immunity By Targeting TRAF3

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**Research**

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# Chicken miR-126-5p negatively regulates antiviral innate immunity by targeting TRAF3

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## Abstract

**Background:** Innate immunity plays an essential role in preventing the invasion of pathogenic microorganisms. However, innate immunity is a double-edged sword, whose excessively activated is detrimental to immune homeostasis and even leads to "cytokine storm" of the infected host. A series of negative regulatory mechanisms are developed by the host to balance the immune response. Here, we report a negative regulatory mechanism of chicken innate immunity mediated by miRNA.

**Results:** In this study we found that the miR-126-5p is markedly up-regulated in RNA virus infected chickens in GEO database. Then, the upregulation of the miR-126-5p by RNA virus was further verified via both cell model and *in vivo* test. Overexpression of miR-126-5p significantly inhibits the expression of interferon related genes and inflammatory cytokines evoked by RNA virus. The opposite result was achieved after knocking down miR-126-5p expression. Bioinformatics analysis indicated TRAF3 as the candidate target gene of miR-126-5p, and experimental evidence, such as the effects of miR-126-5p on the endogenous expression of TRAF3, and the effect of miR-126-5p on TRAF3 3'UTR drove luciferase reporter assay, were provided to further verify that miR-126-5p targets TRAF3. Furthermore, we demonstrated that miR-126-5p negatively regulates innate immunity by blocking the MAVS-TRAF3-TBK1 axis, with co-expression assay.

**Conclusion:** Our results suggest that miR-126-5p is involved in the negative regulation of the chicken innate immunity, which might contribute to maintaining immune balance.

**Key words**

Chicken; miR-126-5p; TRAF3; RNA virus; innate immunity

## Introduction

Innate immunity system as the first line of host defense against pathogenic microorganisms. During viral or bacterial infections, the innate immune system through a series of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), and retinoic acid-inducible gene I (RIG-I) - Like receptor (RLR), the nucleotide-binding oligomerization domain (NOD)-like receptor family proteins (NLRs), to recognize specific pathogen-associated molecular patterns (PAMPs) and cause the release of type I interferons (IFNs) and inflammatory cytokines, trigger antiviral innate immune response [1, 2].

RNA viruses such as influenza A virus (IAV), Avian influenza virus (AIV) and Newcastle disease virus (NDV) cause serious diseases in humans and animals, especially chickens, which have a high mortality rate after infection[3]. TRLs, RLRs and NLRs are the three important influenza virus PRRs for the host. They are distributed on the surface and in the cytoplasm of cells. RLRs include retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2)[4]. However, chicken lacks RIG-I, and chMAD5 plays more important role in recognizing RNA viruses, compared with mammalian MDA5[5, 6]. In mammals, during virus infection, RIG-I and MAD5 interaction with virus RNA, and recruit tumor necrosis factor receptor associated factor 3 (TRAF3) through the adaptor protein MAVS, TRAF3 also as an adaptor protein to phosphorylate TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor-κB (IκB) kinase (IKK) to dephosphorylate the transcription factor IRF3 into the nucleus to activate the production of type I interferon[7, 8]. In birds, IRF3 is also missing, but they have IRF7, and the structure of avian IRF7 is similar to IRF3, and it also activates the secretion of type I interferon[9, 10]. Virus infection will cause a "cytokine storm", which is utilized to control early acute infections and

induce antigen-specific immune responses to viral infections, but it may also cause tissue damage and even death in patients[11, 12]. "Cytokine storm" is caused by the sudden and sharp increase in circulating levels of various pro-inflammatory cytokines including IL-6, IL-8, IL-1β, TNF-α and interferon. Among them, IL-1β activates the release of IL-6, IL-8 and other pro-inflammatory cytokines, and plays an important role in host immunity and epidemic prevention[13, 14].

MiRNAs are small non-coding RNAs that regulate gene expression directly binding to mRNA and influencing translation efficiency[15]. Studies have demonstrated that miRNAs have unique expression profiles in innate and adaptive immune cells, such as monocytes, macrophages, dendritic cells (DCs), T and B cells. And miRNAs play key role in the regulation host immune and "Cytokine storm" [16-18]. The evidence shows that miRNAs can also affect the replication and pathogenesis of RNA viruses by directly binding to the RNA virus genome or through virus-mediated changes in the host transcriptome[19]. Generally, during viral infection, viral proteins inhibit pattern recognition receptor recognition or downstream signal cascades to prevent the production of ISGs. However, it has recently been proposed that virus-mediated regulation of IFN signaling cascades by altering host miRNAs level.

In this study, we analyzed the transcriptome data (GSE111868) of chickens infected with Newcastle disease (NDV) and avian influenza (AIV) and found that miR-126-5p is a differentially expressed miRNAs, and RNA viruses such as AIV and NDV infection chicken DF1 cells significantly increase gga-miR-126-5p expression, miR-126-5p in chicken DF1 cells inhibits innate immune and inflammatory cytokines related genes expression, promotes RNA virus replication by targeting TRAF3. At the same time, we also found that chTRAF3 interacts with chMAVS and chTBK1, and overexpression of chTRAF3 significantly activate the innate immunity of chicken. In conclusion,

in this study we found that miR-126-5p promote RNA virus replication by targeting TRAF3.

## Materials and Methods

### Cell culture and virus

Chicken embryonic fibroblast cell line DF1 and human 293T cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS and cells were incubated at 37 ℃ in a 5% CO2 incubator. Newcastle disease virus (NDV) strain Herts/33 was obtained from the China Institute of Veterinary Drug Control (Beijing, China). Avian influenza virus (AVI). The A/Chicken/Shanghai/010/2008 (H9N2) virus (SH010) was isolated from chicken in Shanghai, China, in 2008 and identified as H9N2 avian influenza A virus. The viruses were purified, propagated, and stored as described in our previous study[20].

### Animal and treatment

Eighteen 3-weeks-old SPF were purchased from the Shanghai Academy of Agricultural Sciences. All chicken were kept in negative-pressure-filtered air isolators and fed as recommending. After one week of adaptation. Eighteen SPF chickens were randomly divided into three groups: the control group (control); the Newcastle disease virus infected group (NDV) and the avian influenza virus infected group (AIV). The virus was diluted with phosphate buffer solution (PBS) and inoculated by eye and nose drops. The AIV group was inoculated with $10^4$ $EID_{50}$ AVI virus, the NDV group was inoculated with $10^5$ $EID_{50}$ NDV virus, and the control group was inoculated with 200 ul PBS. Three days after the inoculation, the chickens in each group were stunned with ether and killed. The spleens, livers, kidney, trachea, bursa of fabricius of these animals were collected, then immediately frozen in liquid nitrogen and stored at −80 ℃ for later use.

### Construction of plasmid

The TRAF3 overexpression vector was constructed by using a pcDNA3.1 according to

manufacturer's instructions. Using the DF1 cells RNA, reverse transcription into cDNA, NCBI to find the chicken TRAF3 sequence (NC_006092.5), primers were designed to amplify CDs region fragment. According to the pcDNA3.1(+) vector map. Primer sequence is as following:

TRAF3

Forward primer: TAGTCCAGTGTGGTG*GAATTC*ATGGACACCAGTAAGAAGACA

Reverse primer: AACGGGCCCTCTAGACTCGAGTCAGGGGTCTGGTAGATCCGA

**Cell transfection**

DF1 were seeded in 12-well or 6-well plates and transfected with miR-126-5p mimics or mimics control (NC), miR-126-5p inhibitor or inhibitor control (NA) (GenePharma, Shanghai, China) at 100 nmol/L was performed with Lipofectamine 2000 according to the manufacturer's protocol.

**Bioinformatics Screening and miR-324-5p Target Gene Prediction**

The RNA virus infection chicken differential miRNA microarray dataset GEO: GSE111868 was initially downloaded from the NCBI GEO portal (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE111868). We systematically analyzed differentially expressed miRNAs (DEGs) of NDV F48E9, La Sota infection chicken embryos using the R package limma. |Log fold change (FC)| >2 and a p value <0.05 were set as the threshold to screen out the DEGs. The online software sanger box (http://sangerbox.com/Tool) was used to draw Ven Maps, Heat Maps and Volcano Maps.

**RNA extraction and qPCR**

Cells total RNAs were extracted with TRLZOL reagent (Takara, Otsu, Japan). mRNA was reverse-transcribed with reverse transcription kits (Takara), and the cDNA analysed using the SYBR green PCR mix (Vazyme, Nanjing, China) with the Applied Biosystems machine (ABI 7500; Thermo Fisher Scientific). Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The β-actin and

U6 small RNA were the internal reference when examining the level of genes and miR-126-5p. The primer sequences for the genes are shown in Table1.

**Dual-luciferase reporter assays**

The wild-type of TRAF3 and the mutant devoid of miR-126-5p binding site were cloned in pmiR-GLO plasmid, construction of pmiR-TRAF3-WT-GLO or mutant pmiR-TRAF3-mutant-GLO plasmid, then cotransfection with miR-126-5p mimics or NC into 293T. At 48h post-transfection, luciferase activities were measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. Primer sequence is as following:

TRAF3-3'UTR

Forward primer: GAGCTCGCTAGCCTCGAGAGGATTTTTGTTTTGTTCTGTT

Reverse primer: CTGCAGGTCGACTCTAGATTTCTAAAGAGAAATAACAGAA

TRAF3-3'UTR- mutant

Forward primer: GTTTCGTGTTCTGCTTTGTAAGAAGATCTTGGA

Reverse primer: AGCAGAACACGAAACAGAGACCAGATGAGGCCTTA

**Coimmunoprecipitation assay and Western Blotting**

The coimmunoprecipitation (Co-IP) was performed as in our previous study. The 293T were seeded in 60 mm dishes, after the cells are more than 80% confluent, they are transfected with 10 μg empty or various expression plasmids. Twenty-four hours post-transfection, the medium was removed and washes twice with ice-cold PBS. Then cells were lysed with 400 mL RIPA Lysis Buffer (Beyotime, Shanghai, China) containing protease cocktail (Yeasen, Shanghai, China) on ice for 30min. Lysates were centrifuged at 14,000xg for 10 min. The supernatant was transfered to a fresh tube and precipitated with 20 μL of anti-Flag or anti-HA immunological

Beads (Bimake, Shanghai, China) for 4h at 4℃. The immunomagnetic beads was washed with cold TBS twice eluted with TBS and 6 x SDS loading buffer (Yeasen, Shanghai, China) by boiling for 10 min. The cell lysates was also eluted with TBS and 6 x SDS loading buffer and boiling. The proteins isolated were separated by SDS-PAGE and analyzed by Western blotting using the indicated antibodies. The images were collected with a Tanon 5200 imaging systems (Tanon, Shanghai, China).

**Statistical analysis**

Results are expressed as the mean ± SD. GraphPad Prism 8.0 was utilized to graph the results. Data were analyzed by Student's t test. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered highly statistically significant (*$p < 0.05$; **$p < 0.01$).

## Results

### The expression of miR-126-5p is upregulated by RNA virus in chicken

MiRNAs are a kind of small non-coding RNA that regulation of many biological processes in cells. A large number of studies have demonstrated that miRNAs also influence virus replication. In order to explore the miRNAs that that affect virus replication, we analyzed the microarray dataset GEO (GSE111868) and previous research results[21] that chickens infected with Newcastle disease (NDV) and avian influenza (AIV) (Figure.S1A), found that miR-126-5p was significantly up-regulated (Figures.S1B and C). The expression level of the miRNA is closely related to its function. In order to explore the role of miR-126-5p in the process of virus invasion, we tested the expression level of miR-126-5p in different tissues of chickens, and found that the expression of miR-126-5p was the highest in the spleen of chickens and the lowest in the bursa of Fabricius (Figure.1A). The spleen is an immune organ of the body, and miR-126-5p has a high expression level, indicating that miR-126-5p may be related to the immune of chickens. Next, we tested the expression of miR-126-5p in different tissues after chickens were infected with NDV and AIV. It was noted that both NDV and AIV virus infection significantly increase the expression of miR-126-5p (Figure.1B). In order to further explore the changes of miR-126-5p in the process of RNA virus invasion, we used AIV, NDV virus and double-stranded RNA (dsRNA) analogue poly (I:C) which is a simulative stimulus of virus RNA, to infect chicken DF1 cells. Cells at different time points of infection were collected and to detect the expression of miR-126-5p, it was found that NDV and poly(I:C) significantly increase the expression of miR-126-5p after 6h of infection, while AIV, NDV and poly(I:C) significantly increase the expression of miR-126-5p after 24h of infection (Figures.1B, C & D). This suggests that miR-126-5p may be involved in the regulation of host responses to RNA virus

infection or to RNA virus pathogen associated molecular patterns (PAMPs).

**Bioinformatics analyses indicate miR-126-5p involved in the regulation of innate immunity**

To investigate the function of miR-126-5p in chicken innate immunity, we through analysis of miR-126-5p mature seed sequence revealed that miR-126-5p was highly conserved in humans, pigs, cattle, chickens and other species (Figure.2A). This suggests that miR-126-5p played an important role in species evolution. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis the target gene of miR-126-5p revealed that it was involved in the regulation of multiple innate immune-related signal pathways, such as Toll-like receptor signaling pathway, RIG-I like preceptor signaling pathway (Figure.3B). Go analysis unveiled that the majority of genes were participated regulates immune cell differentiation and inflammatory factor secretion (Figure.2C). These implied that miR-126-5p has notable effects in chicken innate immunity.

**Overexpression of miR-126-5p inhibits chicken antiviral innate immunity during NDV infection**

In order to verify that miR-126-5p participate in the regulation of chicken innate immunity, we transfected DF1 cells with miR-126-5p mimics or NC (negative control), by detecting innate immune related genes IFNβ, eukaryotic translation initiation factor 2 alpha kinase 2 (PKR), MX1–myxovirus (influenza virus) resistance 1 (MX1), inflammatory cytokine related genes interleukin 1β (IL-1β), interleukin 6 (IL-6) and Interleukin-8 (IL-8) expression, found that miR-126-5p mimics significantly increase the expression of miR-126-5p (Figure.3A), overexpression of miR-126-5p inhibits the expression of the above genes, but only significantly reduce IFNβ, IL -6 expression (Figures.3B and C). While transfected DF1 cells with miR-126-5p mimics or NC, followed by infection RNA virus NDV for 12 hours (Figure.3D), found that overexpressing of miR-126-5p

markedly inhibit innate immune and inflammatory cytokine related genes expression (Figures.3 E and F). These indicate that RNA virus used miR-126-5p to evade the host's antiviral innate immune response.

**Inhibition of miR-126-5p promoted chicken antiviral innate immunity during NDV infection**

Overexpression of miR-126-5p inhibit the expression of chicken antiviral innate immunity related genes. In order to further prove the role of miR-126-5p in chicken antiviral innate immunity, we through loss of function studies, transfection DF1 cells with miR-126-5p inhibitor or NA (Negative control) and detect the RNA expression of innate immune related genes. It was found that miR-126-5p inhibitor significantly inhibited the expression of miR-126-5p (Figure.4A). And inhibiting of miR-126-5p increased the expression of IFNβ and MX1 and PKR (Figure.4B), and obviously increased the expression of inflammatory cytokine related genes IL-1β, IL-6 and IL-8 (Figure.4C). Similarly, after inhibiting the expression of miR-126-5p infection of RNA virus NDV significantly promote the expression of IFNβ, PKR, MX1, IL-1β, IL-6 and IL-8 (Figures.4D, E & F). These show that inhibition of miR-126-5p promote chicken antiviral innate immunity after RNA virus invasion. These results also point out that miR-126-5p participate regulate chicken antiviral innate immunity.

**Interferon signaling pathway has no role in regulation of the expression of miR-126-5p**

After the virus infection of the host, it activates the interferon signal pathway and promotes the expression of interferon-stimulated genes (ISGs) in response to virus invasion. In this study we found, virus invasion significantly increases the expression of miR-126-5p, and KEGG analysis also found that miR-126-5p participate in the regulation of type I interferon signaling pathway (Figure.2B). These indicating that miR-126-5p maybe an interferon stimulated gene. To prove this speculation, we overexpressed chicken IFNβ in DF1 cells (Figure.5A), and found that

overexpression of IFNβ significantly increased the expression of ISGs, such as PKR and MX1 (Figure.5B), but did not affect the expression of miR-126-5p (Figure.5C), These means that the interferon signaling pathway could not regulate the expression of miR-126-5p, and miR-126-5p was not an ISGs.

**MiR-126-5p promotes RNA virus replication**

Immune-related genes are essential for regulating virus replication. In this study, we found that miR-126-5p regulates the expression of immune-related genes. To investigate the regulator role of miR-126-5p in NDV replication. After we transfecting miR-126-5p mimics or inhibitor into DF1 cells, followed by infection with NDV at a multiplicity of infection 0.01 MOI, then determine the copy number of the virus in the cells and culture medium, by detecting the expression of the NDV NP gene. The expression of NDV NP RNA is a sign of virus replication, was significantly increased in the intracellular and supernatant after overexpression miR-126-5p (Figure.6 A & B), while inhibition the expression of miR-126-5p obviously decreased the NDV NP RNA expression (Figure.6 C & D). At the same time, we used the above supernatant of transfecting DF1 cells with the miR-126-5p mimics, inhibitor or NC, followed by infection with NDV for the measurement of viral load by standard assay, such as 50% tissue culture infective dose ($TCID_{50}$) by infecting DF1 cells. Overexpression miR-126-5p markedly increased NDV viral titer and inhibition of miR-126-5p reduce viral titer as determined by $TCID_{50}$ assay (Figure.6 E & F). Thess result shows that overexpression of miR-126-5p promoting the replication of NDV virus, while inhibition of miR-125-5p inhibiting NDV virus replication. To further confirm these results, we infected DF1 cells with NDV-GFP virus and VSV-GFP virus after overexpression or inhibition of miR-126-5p, and found that overexpression of miR-126-5p increased the fluorescence intensity, while inhibiting miR-

126-5p decreased the fluorescence intensity (Figure.6 G). This suggests that miR-126-5p promotes RNA virus replication.

**MiR-126-5p inhibition chicken antiviral innate immunity by targeting TRAF3**

MiRNA exerts a regulatory function by binding target gene mRNA 3'UTR to inhibit the translation of the target gene. In order to understand the molecular mechanism of miR-126-5p in regulation of chicken antiviral immune responses during the RNA virus NDV infection. We used online target gene prediction software Targetscan and found TRAF3 was a candidate target gene of miR-126-5p (Figure.7A). Overexpression of miR-126-5p significantly inhibits the expression of TRAF3 (Figure.7B), while inhibition of miR-126-5p radically increases the expression of TRAF3 (Figure.7C), indicating that miR-126-5p has a regulatory relationship with TRAF3. MiRNAs bind to target genes to inhibit target gene translation and promote target gene degradation. To verify that miR-126-5p accelerates TRAF3 degradation, we overexpression or inhibition the expression of miR-126-5p, the cells were treated with actinomycin D, an inhibitor of transcription, and the cells were collected after treated for 0, 2, and 8 hours. It was found that overexpression of miR-126-5p significantly accelerate the degradation of TRAF3 (Figure.7D), while inhibition of miR-126-5p observed slow down the degradation of TRAF3 (Figure.7E). these show that miR-126-5p maybe target TRAF3. In order to further verify that miR-126-5p by targeting TRAF3 regulation antiviral immune responses, we used the pmiR-GLO plasmid to construct TRAF3 3'UTR containing miR-126-5p binding sequence pmiR-TRAF3-WT-GLO and mutant pmiR-TRAF3-mutant-GLO plasmids (Figure.7F). pmiR-TRAF3-WT-GLO or pmiR-TRAF3-mutant-GLO and miR-126-5p mimics or NC co-transfected, found that miR-126- 5p mimics significantly inhibit the fluorescent activity of pmiR-TRAF3-WT-GLO plasmid, while without affecting the pmiR-TRAF3-mutant-GLO

(Figure.7G). These results indicate that miR-126-5p inhibition chicken antiviral immune responses by targeting the TRAF3.

**TRAF3 regulation chicken innate immunity**

In order to explore the role of TRAF3 in chicken innate immunity, we detected the expression level of TRAF3 in chicken different tissues, and found that the expression level of TRAF3 in chicken trachea, spleen and bursa of Fabricius was higher than that in the kidney (Figure.8A). Chickens infected with NDV or AIV significantly increase the expression level of TRAF3 in the above organizations (Figure.8B). It shows that TRAF3 relates to chicken innate immunity. In order to further explore the effect of TRAF3 on chicken innate immunity, we transfected DF1 cells with pcDNA-TRAF3 or pcDNA3.1, to detect the expression of genes related to innate immunity (Figure.8C). It is found that overexpression of TRAF3 significantly increase the expression of IFNβ and ISGs genes PKR and MX1 (Figure.8D), as well as the expression of inflammatory cytokine related genes IL-1β (Figure.8E). These results TRAF3 is essential for the innate immunity of chickens.

**MiR-126-5p negatively regulates chicken innate immunity by blocking the MAVS-TRAF3-TBK1 axis**

To understand underlying molecular mechanism of TRAF3 mediated enhanced chicken innate immunity, we used the online prediction software STING to predict the TRAF3 interaction proteins and found that TRAF3 interaction with many immune-related proteins such as TBK1, MAVS and IRF7 (Figure.9A). Studies have found that TRAF3 interacts with MAVS in mammals. MAVS and IRF7 are located upstream and downstream of TRAF3, respectively. In order to verify that miR-126-5p regulates chicken innate immunity by targeting TRAF3, we overexpression MAVS or IRF7

and miR-126-5p mimics to detect immune-related and genetic expression. It is found that overexpression of miR-126-5p with MAVS or IRF7 significantly inhibit the expression of IFNβ, PKR and MX1 (Figures.9C & D). These results indicate that miR-126-5p regulates chicken innate immunity by targeting TRAF3, block the MAVS-TRAF3-TBK1 axis.

**Discussion**

Upon virus infection, the host's innate immunity serves as the first line of defense against the virus. When the viral RNA released in the host cell will be recognized by the host's various pattern recognition receptors(PRRs) and secrete type I interferons, pro-inflammatory factors and chemokines[22]. Type I interferon stimulate the expression of hundreds of ISGs in neighboring cells and induce antiviral status[23]. In this study, we found that RNA viruses such as Newcastle disease virus (NDV) and avian influenza virus (AIV) significantly up-regulate the expression of chicken miR-126-5p. After bioinformatics analysis of the target genes of miR-126-5p, we found that miR-126-5p participate in regulation multiple immune-related signaling pathways. It indicates that miR-126-5p may involves in the chicken innate antivirus immunity. A large number of studies have concluded that miR-126-5p promotes the proliferation and migration of cancer cells such as human rectal cancer, ovarian cancer and mouse aortic aneurysm[24-27]. Cancer is due to immune escape [28, 29], which shows that miR-126-5p is not only involved the immunity of chicken also participates in the immunity of mammal.

The above results indicate that miR-126-5p may be regulated by type I interferon. However, we found chIFNβ significantly up-regulate the expression of two typical ISGs, MX1 and PKR, but did not affect the expression of miR-126-5p, this shows that miR-126-5p is not regulated by IFNβ. More importantly, miR-126-5p inhibits the expression of innate immune and inflammatory cytokines

related genes following NDV virus infection. Similarly, EV71 infection up-regulates the expression of miR-141, resulting in down-regulation of the eukaryotic translation initiation factor 4E protein, thereby promoting EV71 replication and release[30]. These indicate that miR-126-5p regulates host antiviral immunity. But how the host regulates the expression of miR-126-5p after virus invasion still needs further study.

Excessive immune response after virus invasion can cause " cytokine storm ", which severely damage the host's organs and even cause death[12]. A large number of studies have shown that miRNAs participate in the regulation of host cytokine storm after virus invasion. For example, after influenza virus invasion, miR-302a can induce cytokine storm, and miR-133a also has the same effect[31, 32]. Interestingly, we found that miR-126-5p significantly inhibits the expression of inflammatory cytokines related genes after NDV virus infection. At the same time, miR-126-5p notably promotes the replication of NDV virus. This indicates that miR-126-5p regulate the host's immune response by suppressing the host "cytokine storm". This negative regulation can effectively avoid damage to the host by a strong immune response.

miRNAs regulates the protein abundance of target genes by binding and inhibiting the translation of gene mRNA[15]. In order to study the mechanism of miR-126-5p inhibiting chicken antiviral innate immunity, we analyzed the target genes of miR-126-5p. It finds that miR-126-5p targets and inhibits the expression of TRAF3. A large number of studies have shown that TRAF3 plays a significant role in the process of antiviral innate immunity[33-35]. It is a MAVS adaptor protein that undergoes k63-linked ubiquitination in mitochondria during RNA virus infection. This post-translational modification induces two kinds of Iκ-B kinase (IKK) related kinase, TANK binding kinase 1 (TBK1) and IKKi activation. These two kinases phosphorylate interferon regulatory factor 3 (IRF3) or IRF7,

resulting in the type I interferon (IFN)[36, 37]. In this study, we also found that RNA virus infection in chickens significantly increase the expression of TRAF3, and overexpression of TRAF3 enhance the innate antiviral immunity of chicken. Our previous study found that MAVS acts as a scaffold protein to recruit and phosphorylate TBK1 and IRF7 to activate IFNβ[10, 38]. Coupled with this study, we think that MAVS may first recruit TRAF3 and then recruit TBK1 and IRF7. In order to further prove that miR-126-5p inhibits the type I interferon signaling pathway through TRAF3, we separately co-overexpressed the TRAF3 upstream and downstream proteins MAVS and IRF7 with miR-126-5p to detect the relate genes expression of type I interferon signaling pathway. It was found that the simultaneous overexpression of MAVS or IRF7 with miR-126-5p significantly inhibit the expression of IFNβ, MX1 and PKR. This shows that TRAF3 is required for the activation of type I interferon pathways.

However, in this study we also found that miR-126-5p regulate virus replication. Existing research findings the dual regulation role of miRNAs for virus replication. Firstly, miRNAs exert an antiviral effect, and inhibit virus replication by directly binding to the virus 3'NTR. In addition, miRNAs directly bind to the 5'NTR of the virus to stabilize the virus structure and promote virus replication. Of course, viruses can also promote their replication by changing the abundance of host miRNAs[19, 39]. Here, we found that overexpression of miR-126-5p significantly promotes virus replication, while inhibition of miR-126-5p inhibits virus replication. We believe that miR-126-5p regulation of viral replication may be related to miR-126-5p negative regulation of innate immunity.

**Conclusions**

In conclusion, our research determines that miR-126-5p negative regulatory the chicken antiviral innate immunity. After RNA virus infection, up-regulate the expression of miR-126-5p, then it by

targeting TRAF3 inhibiting the type I interferon signaling pathway and inflammatory factors. We also found that miR-126-5p is highly conserved among species, and influenza virus infection with A549 cells also change the expression of miR-126-5p[40]. So it may also participate in the mammalian antiviral immune response. However, the specific role of miR-126-5p in mammalian innate immunity remains unclear. Therefore, our research will provide potential targets for the treatment of cytokine storms caused by viral infections. (Figure.10).

## Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

## Abbreviations

**AIV:** Avian Influenza Virus

**NDV:** Newcastle Disease Virus

**TRAF3:** TNF receptor-associated factor 3

**IFNβ:** Interferon-beta

**PKR:** Eukaryotic translation initiation factor 2 alpha kinase 2

**MX1:** MX1–myxovirus (influenza virus) resistance 1 (MX1)

**IL-1β:** Inflammatory cytokine related genes interleukin 1β

**IL-6:** Interleukin 6

**IL-8:** Interleukin-8

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## Contributions

JW, LLW, and JHS designed the research and analyzed the data. JW, LLW, YQC, ZYL, WXZ, CLZ, WXZ, YXY and JHS conducted the experiments and collected the data. JW and YQC wrote the paper. All authors approved the final version of the manuscript.

## Corresponding author

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## Ethics declarations

Ethics approval and consent to participate

The protocols used in this study were approved by the Animal Research Ethics Committee of Shanghai Jiaotong University. The animal experiments were performed according to Guide for the Care and Use of Laboratory Animals of SJTU.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

**Figure Captions**

**Figure.1** RNA virus upregulates the expression of miR-126-5p in chicken. (A) qPCR analysis of miR-126-5p expression in bursa of fabricate, lung, spleen, trachea, liver and kidney of healthy chickens.; (B) The changes of miR-126-5p expressions in the bursa, lung, spleen, trachea, liver and kidney of chickens after infection with NDV and AIV by qPCR. (C-E) DF1 cells were infected with AIV or NDV or poly(I:C), the expressions of miR-126-5p at 0 h (C), 6 h(D), and 24 h post–infection were detected by qPCR. The data are expressed as mean ± SD; n = 3. *$p < 0.05$, **$p < 0.01$.

**Figure.2** Bioinformatics analyses indicate miR-126-5p involved in the regulation of innate immunity. (A) Sequence alignment of miR-126-5p from various vertebrate species. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of miR-126-5p target genes. (C) Gene Ontology (GO) term analysis of miR-126-5p target genes.

**Figure.3** Overexpression of miR-126-5p inhibits chicken antiviral innate immunity during NDV infection.(A) qPCR analysis of miR-126-5p overexpression efficiency, after miR-126-5p mimics or NC (Negative control) transfection of DF1 cells 24h. (B)qPCR detection the RNA level of innate immune-related genes, including IFNβ, ISGs, PKR and MX1 expression after overexpression of miR-126-5p. (C) qPCR detection of inflammatory cytokine related gene RNA level, such as IL-Iβ、IL-6 and IL-8 expression after overexpression of miR-126-5p. (D-E) Transfection DF1 cells with miR-126-5p mimics or NC, followed by infection RNA virus NDV for 12 hours. (D) qPCR analysis of miR-126-5p overexpression efficiency. (E) qPCR detection of IFNβ and ISGs PKR and MX1 expression level. (F) qPCR detection of inflammatory cytokine related gene IL-Iβ、IL-6 and IL-8 expression level. The data are expressed as mean ± SD; n = 3. *$p < 0.05$, **$p < 0.01$.

**Figure.4** Inhibition of miR-126-5p promoted chicken antiviral innate immunity during NDV

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624 NA(Negative control) transfection of DF1 cells 24h. (B) qPCR detection the RNA level of innate
625 immune-related genes, including IFNβ, ISGs, PKR and MX1 expression after inhibitor of miR-126-
626 5p. (C) qPCR detection of inflammatory cytokine related gene RNA level, such as IL-Iβ、IL-6 and
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633 (A) After transfection of pcDNA-IFNβ into DF1 cells, the overexpression efficiency of IFNβ was
634 detected by quantitative real-time PCR. (B) qPCR detection the expression of ISGs PKR and MX1
635 after overexpression of IFNβ. (C) qPCR detection the expression of miR-126-5p after
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638 miR-126-5p mimics or NC, followed by infection with NDV at MOI of 0.01 after 6h, intracellular
639 and supernatant relative levels of NP RNA were measured by absolute quantitative real-time PCR
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643 126-5p on NDV replication by $TCID_{50}$. (G) NDV-GFP or VSV-GFP infected DF1 cells visualized
644 48 hours post-transfection and post-infection using fluorescence microscopy and representative

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**Figure.8** TRAF3 can activate chicken innate immunity. (A) qPCR analysis of TRAF3 expression in in chicken bursa of Fabricius, lung, spleen, trachea, liver and kidney tissues; (B) qPCR detection the change of TRAF3 in the bursa, lung, spleen, trachea, liver and kidney tissues after chickens infected with NDV and AIV. (C) After transfection of pcDNA-TRAF3 into DF1 cells, the overexpression efficiency of TRAF3 was detected by qPCR. (D) qPCR detection the expression of IFNβ and ISGs PKR and MX1 after overexpression of TRAF3. (E) qPCR detection of inflammatory cytokine gene IL-Iβ、IL-6 and IL-8 expression after overexpression of TRAF3. The data are expressed as mean ± SD; n = 3. *$p < 0.05$, **$p < 0.01$.

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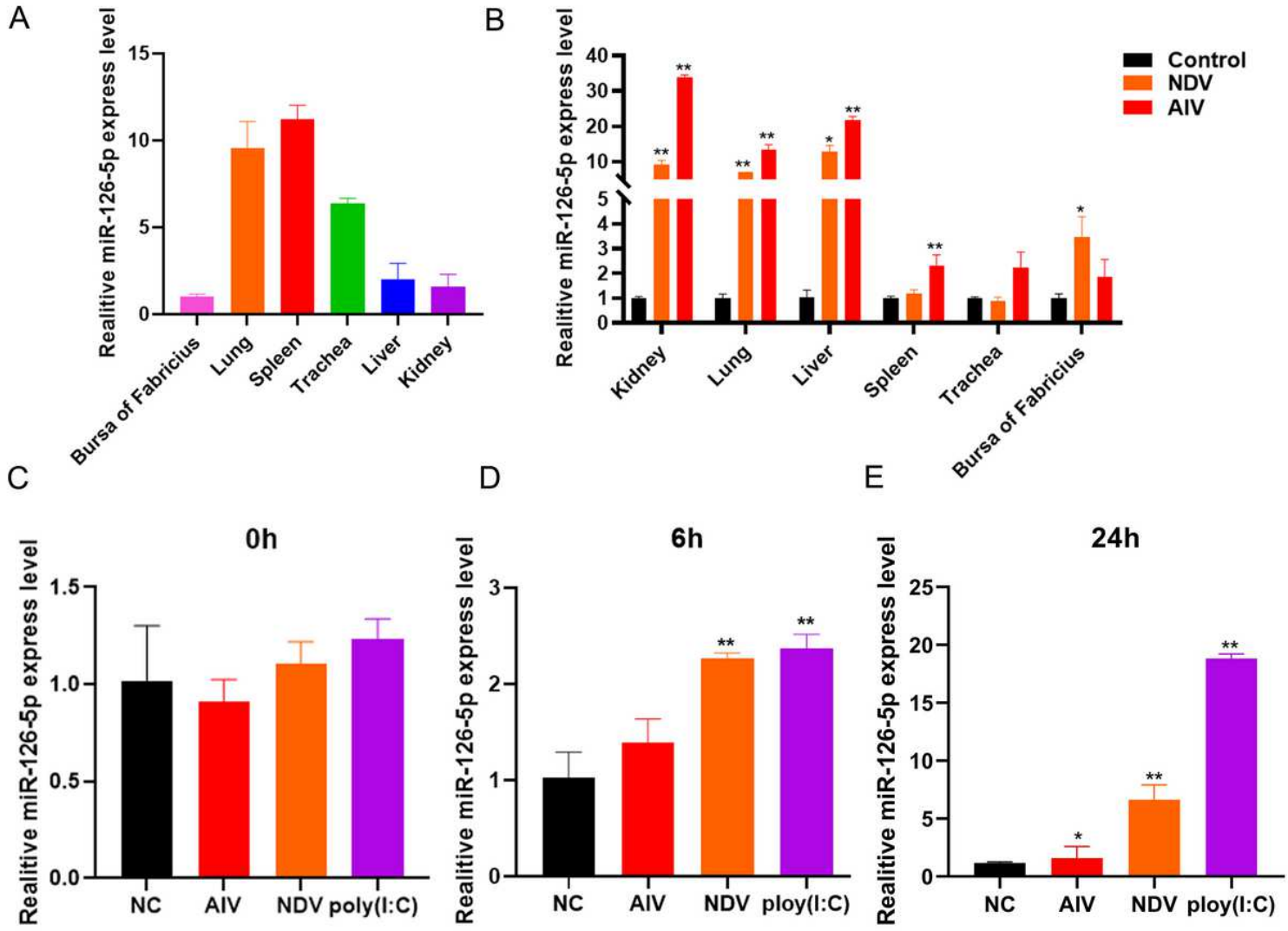
**Figure.10** Schematic illustration of miR-126-5p serving as immune rheostat by targeting TRAF3.

# Figures

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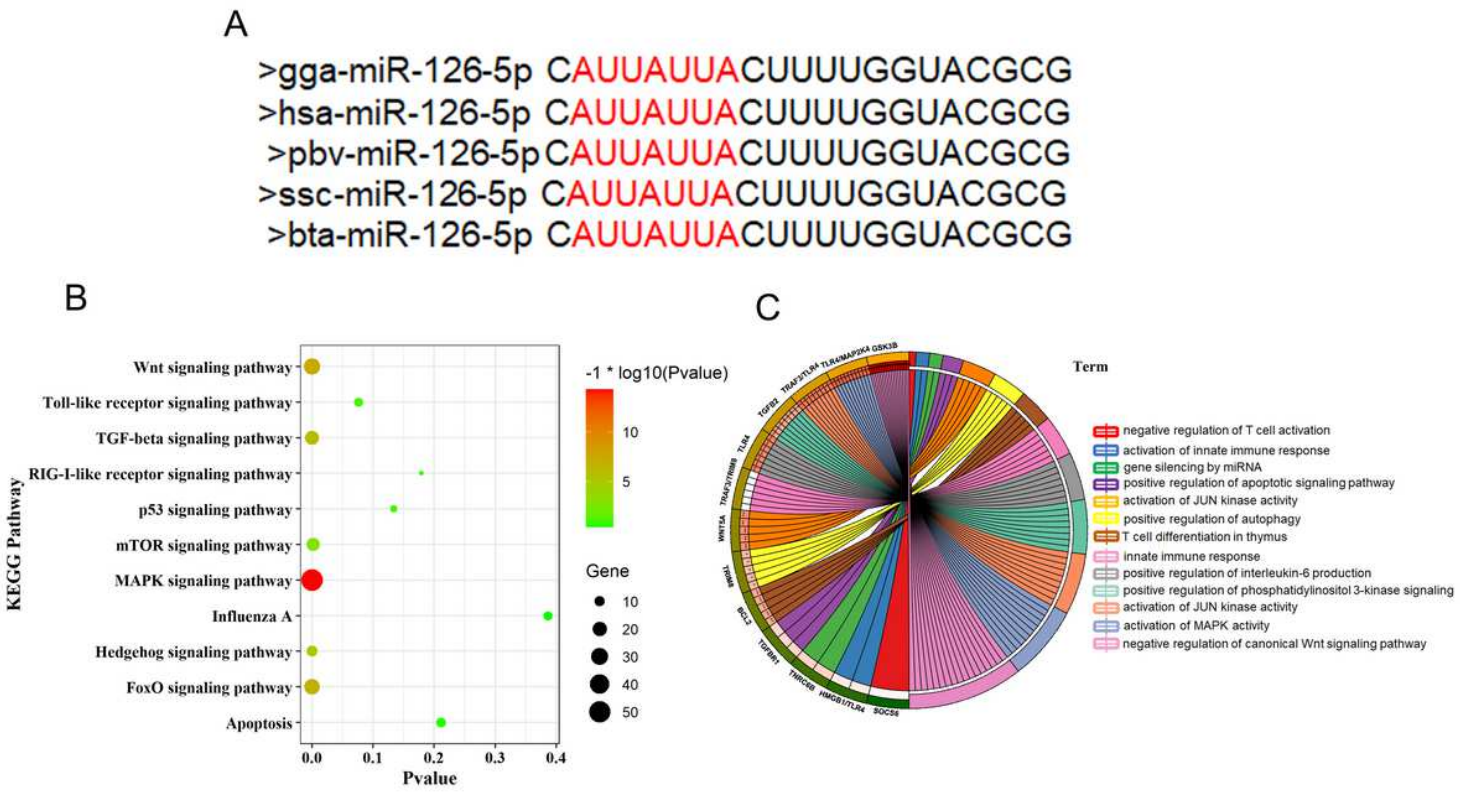


## Figure 2

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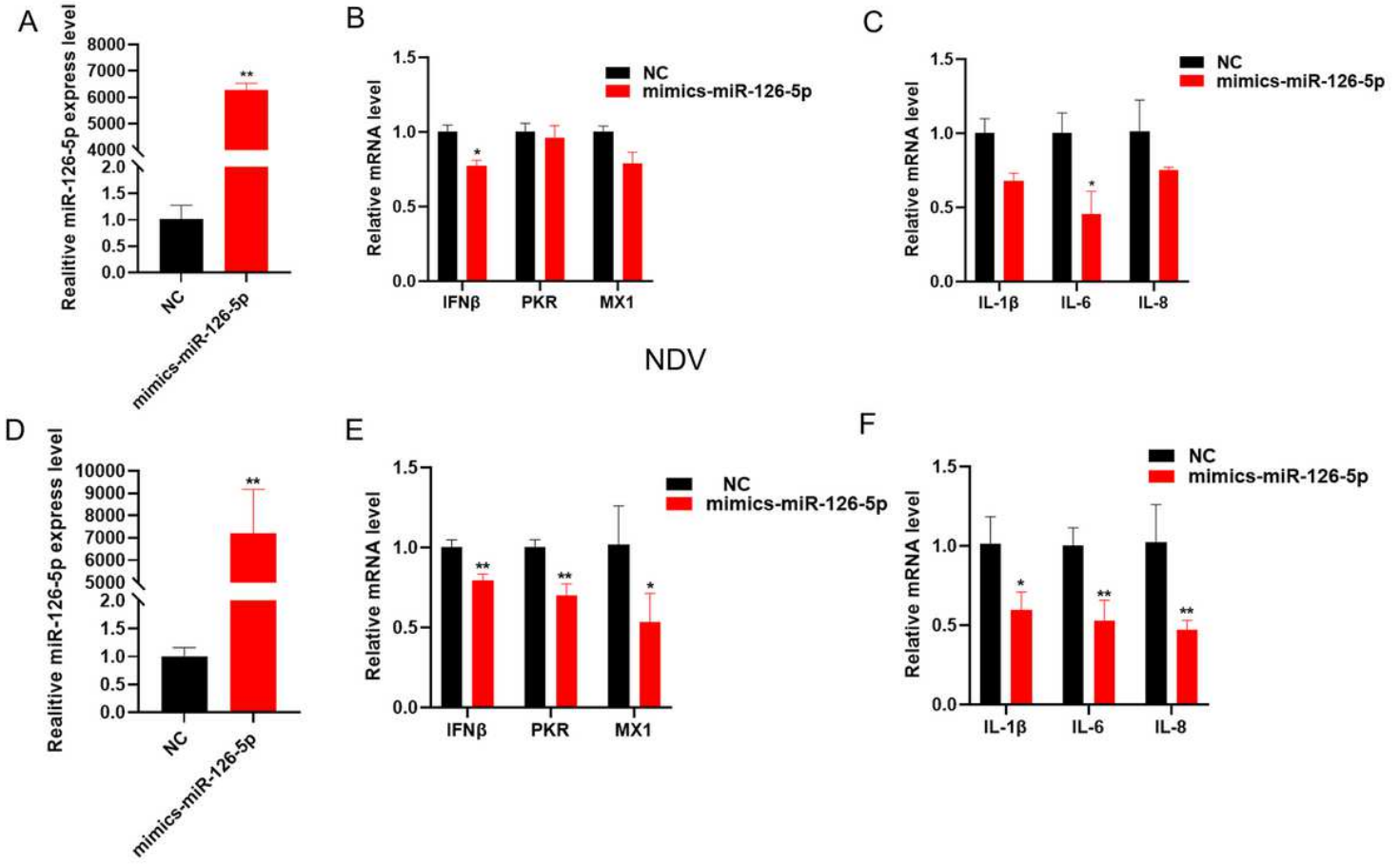


## Figure 3

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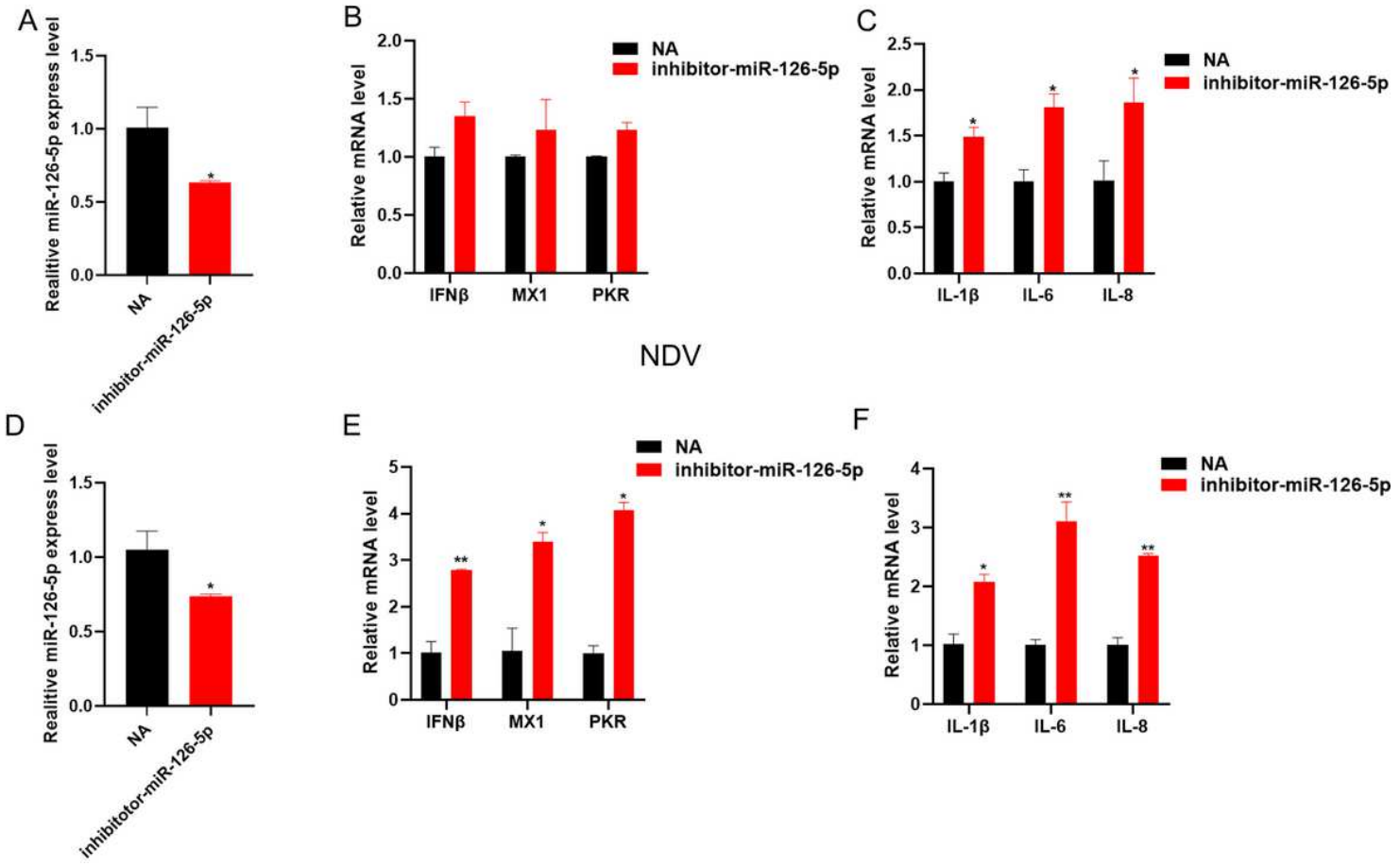
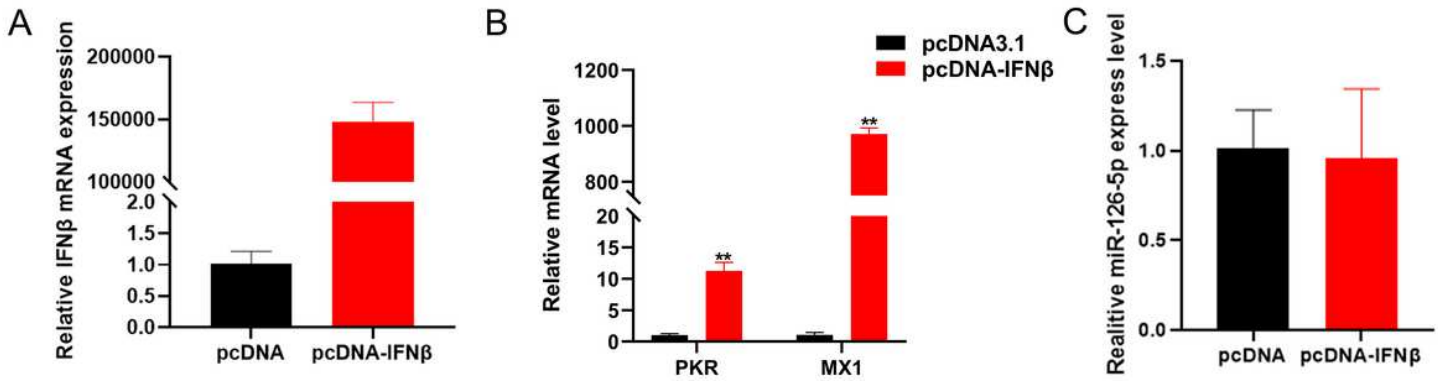



## Figure 4

Inhibition of miR-126-5p promoted chicken antiviral innate immunity during NDV infection. (A) qPCR analysis of miR126 5p inhibition efficiency, after miR-126-5p inhibitor or NA(Negative control) transfection of DF1 cells 24h. (B) qPCR detection the RNA level of innate immune-related genes, including IFNβ, ISGs, PKR and MX1 expression after inhibitor of miR-126-5p. (C) qPCR detection of inflammatory cytokine related gene RNA level, such as IL-Iβ IL-6 and IL-8 expression after inhibitor of miR-126-5p. (D-E) Transfected DF1 cells with miR-126-5p inhibitor or NA, followed by infection RNA virus NDV for 12 hours. (D) qPCR analysis of miR126 5p inhibition efficiency. (E) qPCR detection of IFNβ and ISGs PKR and MX1 expression level. (F) qPCR detection of inflammatory cytokine related gene IL-Iβ IL-6 and IL-8 expression level. The data are expressed as mean ± SD; n = 3. *p < 0.05, **p < 0.01.



## Figure 5

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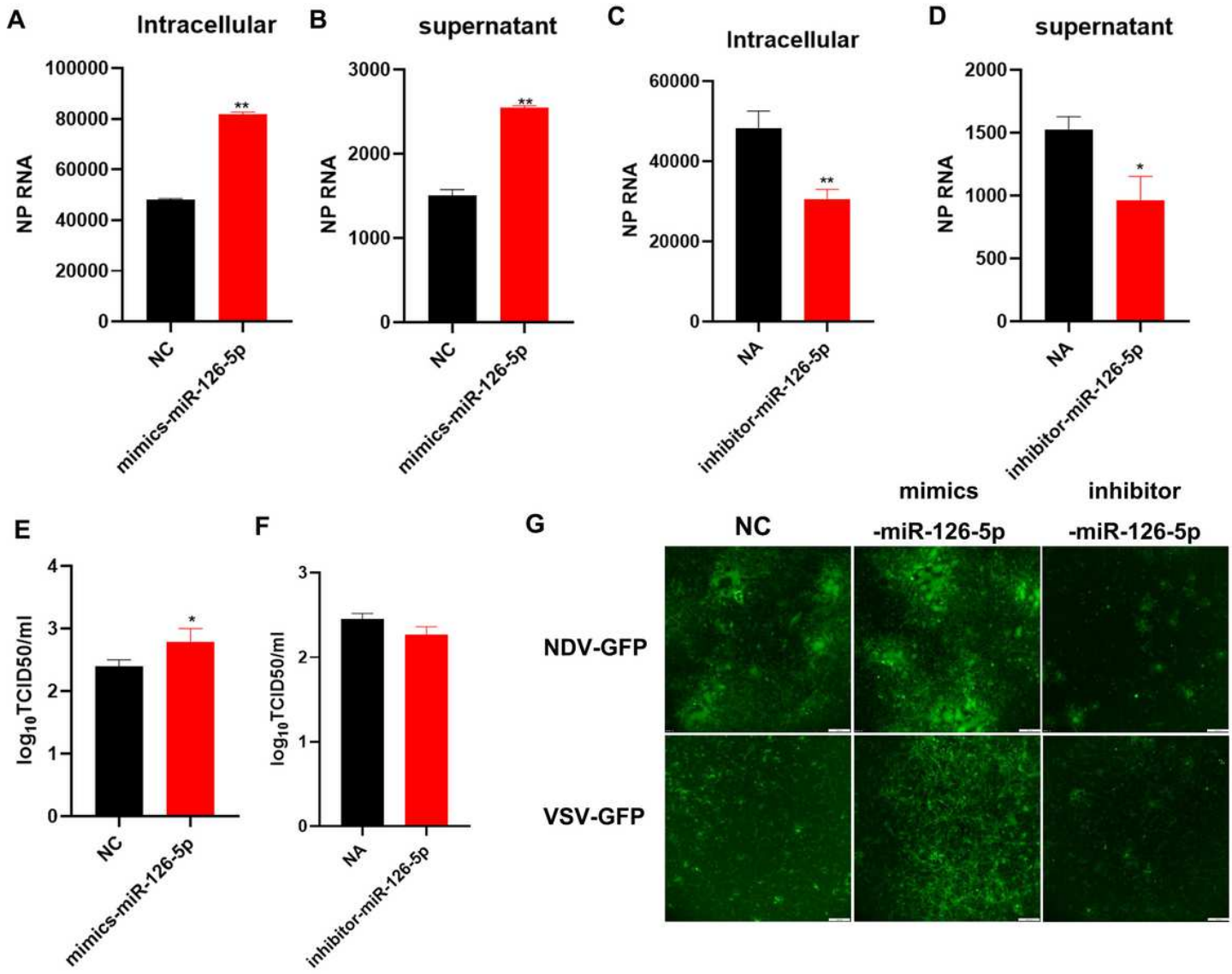


## Figure 6

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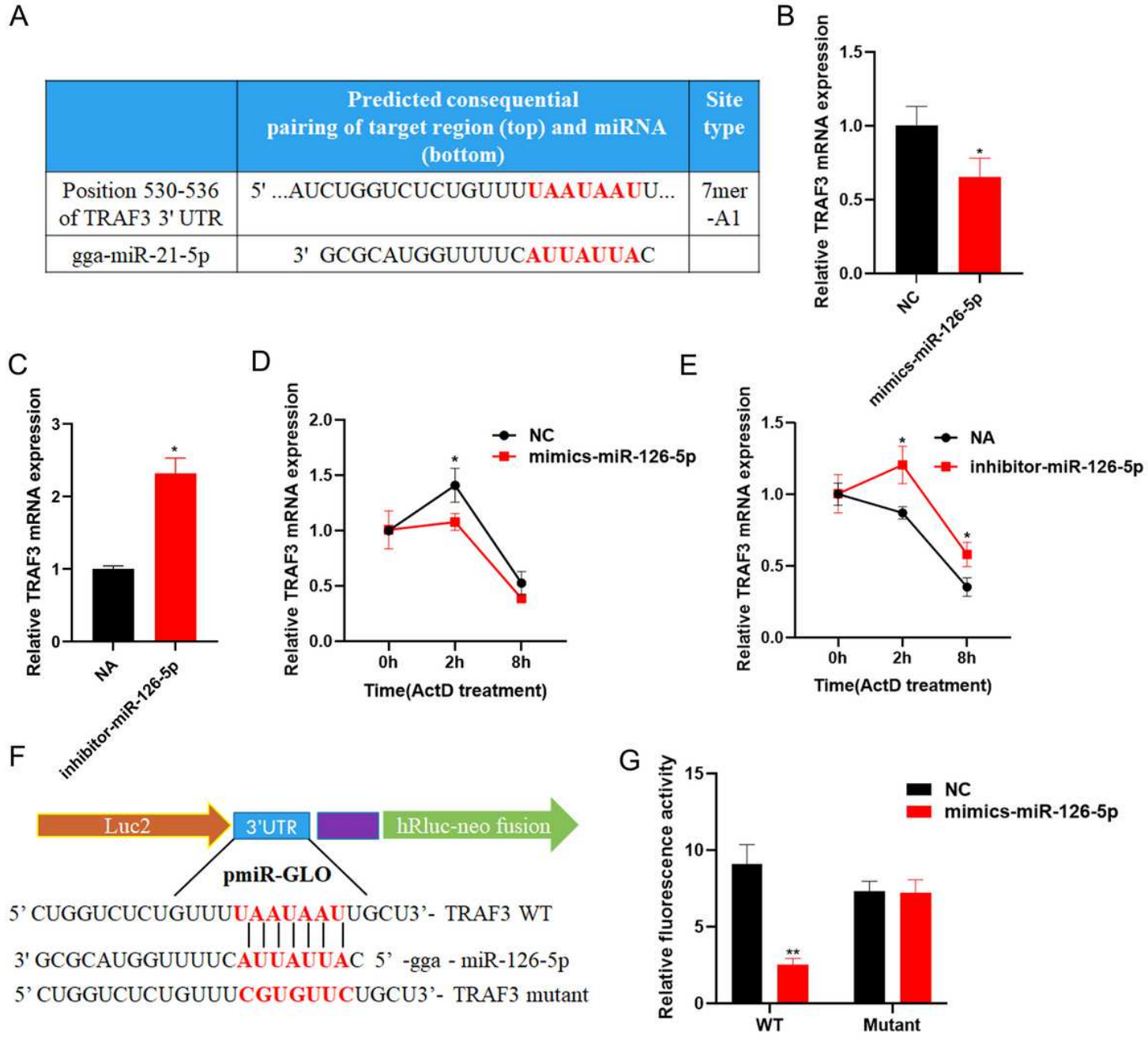


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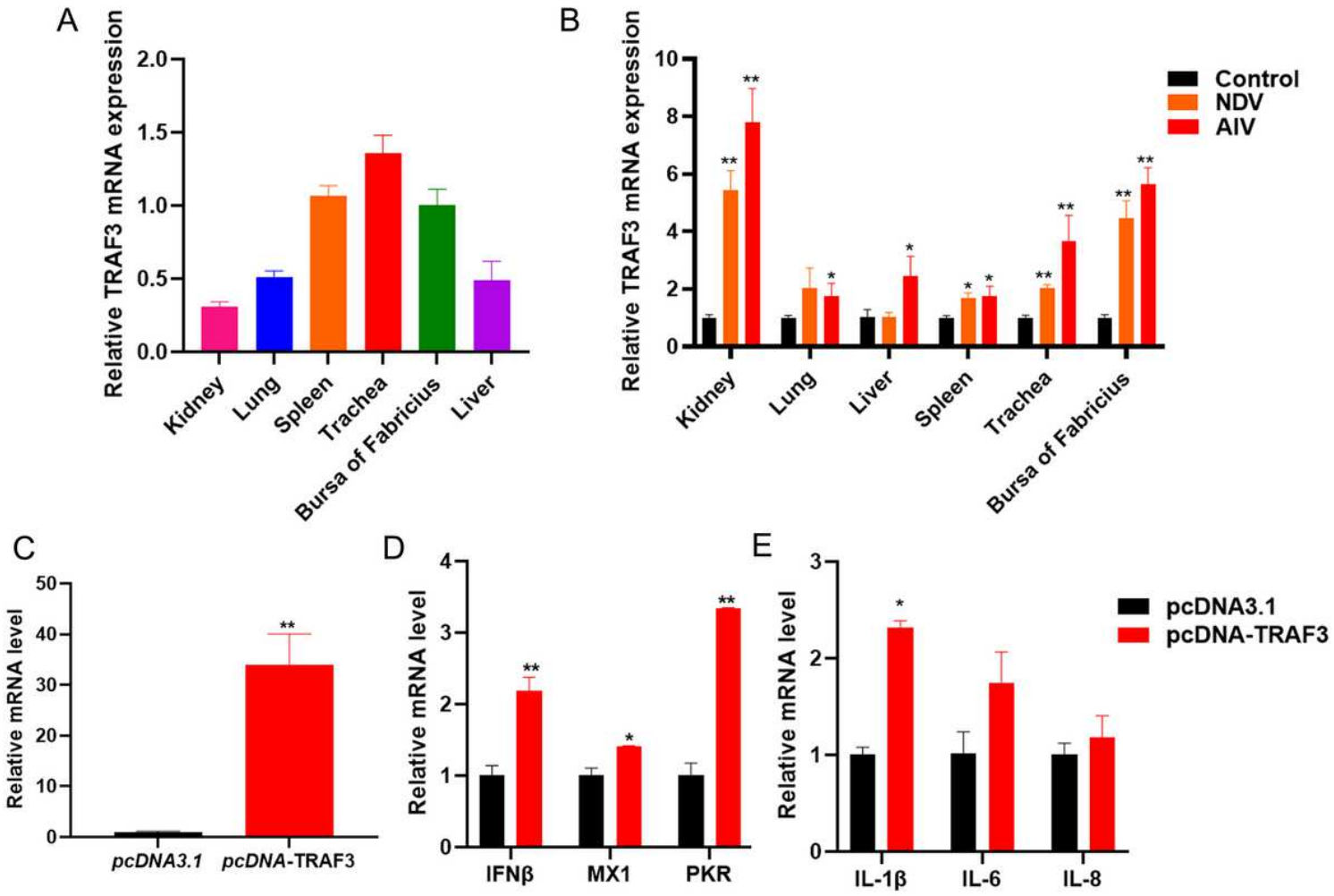


## Figure 8

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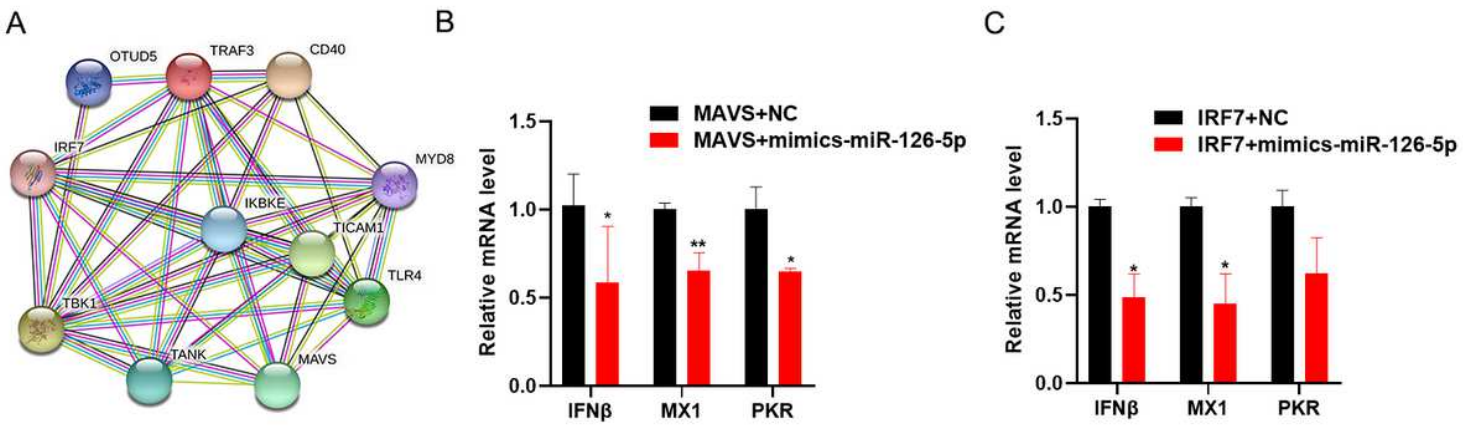


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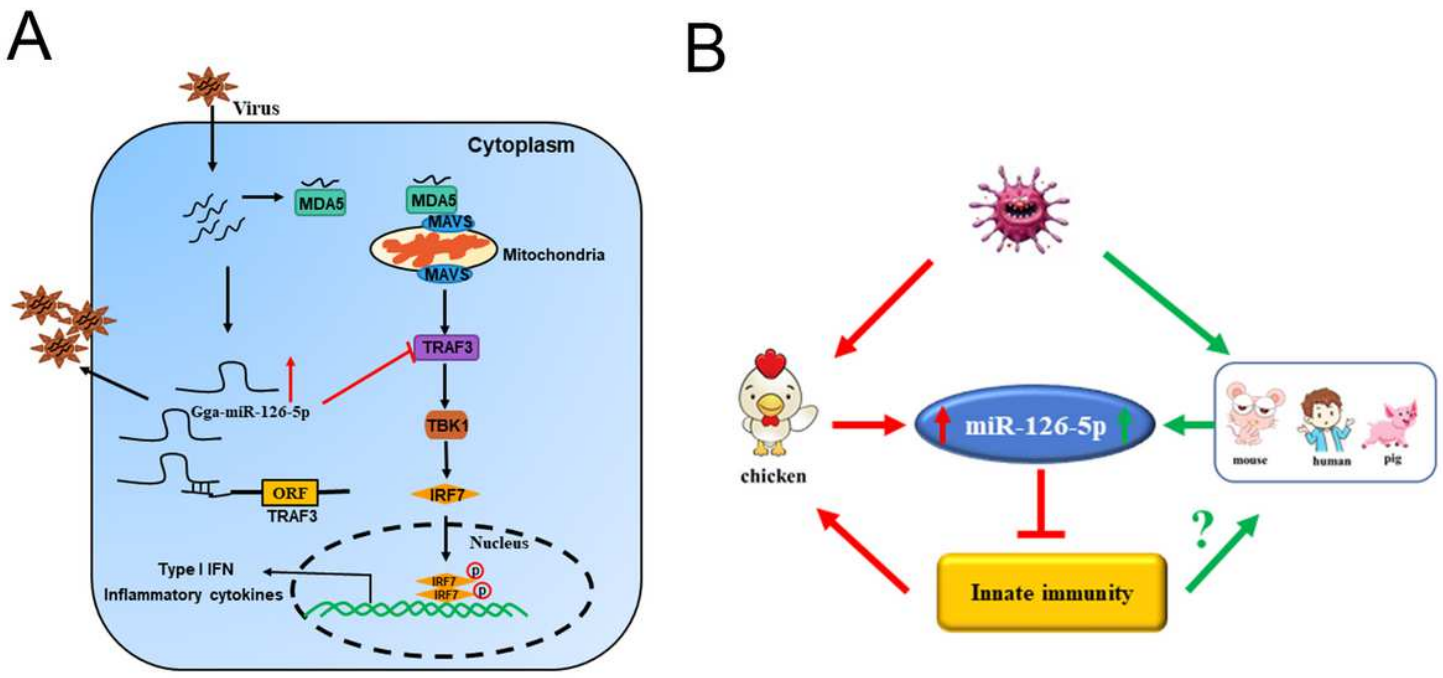


**Figure 10**

Schematic illustration of miR-126-5p serving as immune rheostat by targeting TRAF3.

## Supplementary Files

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