Caspase-dependent cleavage of DDX21 suppresses host innate immunity

Wei Wu¹, Yang Qu², Shengqing Yu¹, Sa Wang³, Yuncong Yin⁴, Qinfang Liu¹,
Chunchun Meng¹, Ying Liao¹, Zaib Ur Rehman¹, Lei Tan¹, Cuiping Song¹, Xusheng
Qiu¹, Weiwei Liu¹, Chan Ding^{1,4*}, Yingjie Sun^{1*}

Running head: DDX21 cleavage suppresses innate immunity

1 Department of Avian Infectious Diseases, Shanghai Veterinary Research Institute,
Chinese Academy of Agricultural Science, Shanghai, 200241, P.R. China

2 College of Veterinary Medicine, Northwest A&F University, Yangling, 712100,
Shaanxi, PR China

3 College of Animal Sciences, Fujian Agriculture and Forestry University, Fuzhou
350002, P.R. China

4 Jiangsu Co-innovation Center for Prevention and Control of Important Animal
Infectious Diseases and Zoonoses, Yangzhou, 225009, P.R. China

* Equal corresponding Author:

Address: 518 Ziyue Road, Shanghai 200241, P. R. China

Phone: +86-21-34293441

Fax: +86-21-54081818

E-mail address: sunyingjie@shvri.ac.cn; shoveldeen@shvri.ac.cn

Abstract: DEAD (Glu-Asp-Ala-Glu)-box RNA helicases have been proven to contribute to antiviral innate immunity. DDX21 RNA helicase was identified as a nuclear protein involved in ribosomal RNA processing and RNA unwinding. DDX21 was also proved to be the scaffold protein in the complex of DDX1-DDX21-DHX36 which senses double strand RNA and initiates downstream innate immunity. Here, we identified that DDX21 undergoes caspase-dependent cleavage after virus infection and treatment with RNA/DNA ligands, especially for RNA virus and ligands. Caspase-3/6 cleave DDX21 at D¹²⁶ and promotes its translocation from the nucleus to the cytoplasm in response to virus infection. The cytoplasmic cleaved DDX21 negatively regulates the IFN- β signaling pathway by suppressing the formation of DDX1-DDX21-DHX36 complex. Thus, our data identify DDX21 as a regulator of immune balance and most importantly uncover a potential role of DDX21 cleavage in the innate immunity response towards virus.

Keywords: DDX21, virus infection, cleavage, innate immunity

36 **Importance**

37 Innate immunity serves as the first barrier against virus infection. DEAD
 38 (Glu-Asp-Ala-Glu)-box RNA helicases, originally considered to be involved RNA
 39 processing and RNA unwinding, have been shown to play an important role in
 40 anti-viral innate immunity. The precise regulation of innate immunity is critical for the
 41 host because the aberrant production of cytokines leads to unexpected pathological
 42 consequences. Here, we identified DDX21 was cleaved at D¹²⁶ by virus infection and
 43 treatment with RNA/DNA ligands via the caspase-3/6-dependent pathway. The
 44 cytoplasmic cleaved DDX21 negatively regulates the IFN- β signaling pathway by
 45 suppressing the formation of DDX1-DDX21-DHX36 complex. In sum, our data
 46 identify DDX21 as a regulator of immune balance and most importantly uncover a
 47 potential role of DDX21 cleavage in the innate immunity response towards virus.
 48

49 **Introduction**

50 The host innate immunity response is initiated by virus infection. Several pathogen
51 recognition receptors (PRRs) were mobilized to sense viral nucleic acids and
52 ultimately lead to the induction of interferons (IFNs) and other inflammatory
53 cytokines to protect host cells (1). Among them, endosomal toll-like receptor 3 (TLR3)
54 (2, 3), cytoplasmic retinoic acid-inducible gene I (RIG-I), and melanoma
55 differentiation-associated protein 5 (MDA5) (4, 5) were demonstrated to be critical
56 for sensing viral double-stranded RNA (dsRNA). The adaptor protein TIR
57 domain-containing adaptor inducing interferon- β (TRIF) (2) and mitochondrial
58 protein MAVS (VISA, IPS-1, or Cardif) (6-9) then activated, leading to the activation
59 of nuclear factor kappa B (NF- κ B) and transcription factor interferon regulatory factor
60 3 (IRF3) as well as the production of various cytokines, including type I-IFNs (10).
61 The secreted IFN binds to the IFN receptor and induces the expression of various
62 interferon-stimulated genes (ISGs) to establish a cellular anti-viral state (1).

63 The helicase family is a class of enzymes that are essential to all living
64 organisms. Their main function is separating nucleic acid strands (DNA, RNA, or
65 RNA:DNA hybrid) (11). The human genome encodes 64 RNA helicases and 31 DNA
66 helicases, which are classified in several superfamilies (SFs) based on their conserved
67 motifs (12, 13). DEAD/H box helicase belongs to the SF2 family, the largest group of
68 helicases, which are involved in various cellular processes (12, 13). RNA helicases
69 are critical for most RNA metabolism processes and are also involved in the antiviral

immune response by sensing foreign RNAs (14). RIG-I, MDA5, and laboratory of genetics and physiology 2 (LGP2), three RIG-I-like receptors (RLRs) that belong to SF2 RNA helicases, are closely related to DEAD-box helicases (15). In addition to RLRs, a growing list of DEAD/H box helicases has been identified to contribute to antiviral innate immunity in recent years, either by acting as sensors for viral nucleic acids or as mediators of downstream signaling events (16-21).

DDX21 was shown to be an abundant nuclear protein in HeLa cells that directly binds rRNAs and snoRNAs and promotes rRNA transcription, processing, and modification (22-25). Another important function of DDX21 is unwinding RNAs, including dsRNA and RNA guanine quadruplexes (26-28). Recently, several reports indicated that DDX21 also plays a role in innate immunity and virus infection. DDX21, together with DDX1 and DHX36, can bind the adaptor protein TRIF to sense dsRNA (20). During dengue virus infection, DDX21 translocates from nucleus to cytoplasm and mediates innate immunity (29). Moreover, DDX21 inhibits influenza A virus replication but is counteracted by viral NS1 protein (30). However, the way in which DDX21 precisely regulates anti-viral innate immunity and whether DDX21 undergoes protein modification during virus infection remain unclear.

Our preliminary screening results showed that Newcastle disease virus (NDV) manipulates the expression of several DDX/DHXs during infection (data not shown). Among them, DDX21 was found to be cleaved in HeLa cells infected with NDV. Here, we report that virus infection and RNA/DNA ligands cleave DDX21 at D¹²⁶ via

91 the caspase-3/6 pathway. The cleavage of DDX21 promotes its translocation from the
 92 nucleus to the cytoplasm in response to virus infection. The cytoplasmic cleaved
 93 DDX21 negatively regulates the IFN- β signaling pathway by suppressing the
 94 formation of DDX1-DDX21-DHX36 complex. Our study therefore reveals a role of
 95 DDX21 in the regulation of anti-viral innate immunity and provides molecular
 96 insights into how the host balances anti-viral and aberrant innate immunity.

97 **Materials and Methods**

98 **Reagents and antibodies**

99 Caspase inhibitor z-VAD-FMK (C1202, Beyotime, Nantong, China) was used at a
100 concentration of 50 μ M. Neddylation inhibitor MLN4924 (S7109, Selleckchem,
101 Houston, TX, USA) was used at a concentration of 1 μ M. Proteasome inhibitor
102 MG-132 (S1748, Beyotime) was used at a concentration of 20 μ M. The autophagy
103 inhibitors wortmannin (W1628, Sigma-Aldrich, St. Louis, MO, USA) and
104 chloroquine (CQ) (C6628, Sigma-Aldrich) were used at 300 nM and 25 μ M
105 concentrations, respectively. Poly(I:C) (tlrl-pic), 3p-hpRNA (tlrl-hprna), HSV-60
106 (tlrl-hsv60n) and poly(dG:dC) (tlrl-pgcn) were purchased from Invivogen (San Diego,
107 CA, USA). Rabbit monoclonal anti-DDX21 (ab182156), anti-caspase-6 (ab185645),
108 mouse monoclonal anti-VSV-glycoprotein (G) antibodies (ab50549), and rabbit
109 polyclonal anti-Lamin B1 antibodies (ab16048) were purchased from Abcam
110 (Cambridge, MA, USA). Rabbit polyclonal anti-HSV-1-glycoprotein D (gD)
111 (NB600-516) was purchased from Novus biologicals (Littleton, CO). Rabbit
112 polyclonal anti-Sendai (PD029C1) was purchased from MBL (Nagoya, Japan). Rabbit
113 polyclonal anti-caspase-3 (GTX110543) was purchased from GeneTex (Irvine, CA).
114 Mouse monoclonal anti-Flag (F1804), anti-HA (H9658), and anti- β -actin antibody
115 (A1978) were purchased from Sigma-Aldrich. Rabbit monoclonal anti-phospho-
116 TANK Binding Kinase 1 (TBK1) (#5483) and anti-TBK1 (#3013) were purchased
117 from Cell Signaling Technology (Beverly, MA, USA). Monoclonal antibody against

NDV nucleoprotein (NP) was prepared in our laboratory (31). For the immunofluorescence assays, mouse monoclonal anti-HSV-1/2 gE was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and mouse polyclonal anti-VSV-G antibodies (ab1874) were purchased from Abcam. IFN- β was measured with an ELISA kit (41410, PBL Assay Science, Piscataway, NJ).

Cell cultures and virus

HeLa, A549, HEK-293T, Vero, HuH7, and THP-1 cells were purchased from American Type Culture Collection (ATCC). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). NDV Herts/33 strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China). Herpes simplex virus (HSV)-1 was kindly provided by Yasushi Kawaguchi (University of Tokyo, Japan), and vesicular stomatitis virus (VSV) was provided by Jianchao Wei (Shanghai Veterinary Research Institute, China). Sendai virus (SeV) was provided by Quan Zhang (Yangzhou University, China). HSV-1 and VSV titration was determined as the median tissue culture infective dose (TCID₅₀) on Vero cells.

Plasmids

Flag-tagged DDX21-X1 (Flag-DDX21) and DDX21-X2 were constructed by inserting the open reading frame (ORF) of human DDX21 isoform 1 (NM_004728.4) and isoform 2 (NM_001256910.1) into plasmid p3XFLAG-CMV-14 (Sigma-Aldrich),

respectively. Flag-tagged deletion constructs (Δ 217-395, Δ 396-573) and point mutants of DDX21 (D87A, D126A, 160A, D87A/D126A, D87A/160A, D126A/D160A, D81A/D126A/160A) were generated by site-directed mutagenesis, as described previously (32, 33). Flag- and HA-tagged wide-type and truncates of DDX21 (1-125, 127-784, Δ 1-216, Δ 574-784) were constructed by inserting indicated sequences into p3XFLAG-CMV-14 (Sigma-Aldrich) and pCMV-HA (Promega), respectively. HA-tagged DDX1 and HA-tagged TRIF were constructed by inserting indicated sequences into pCMV-HA (Promega), respectively. Myc-tagged DHX36 was constructed by inserting the ORF of DHX36 into pCMV-Myc (Promega). pHAGE-WT and -D126A DDX21 were constructed by inserting the flag-tagged WT and D126A DDX21 into pHAGE-bsd, which was constructed based on pHAGE-puro (Addgene, plasmid 118692). The primer sequences for plasmid construction are listed in Supplementary Table 1. The IFN- β promoter luciferase reporter was kindly provided by Takeshi Fujita (Kyoto University, Japan).

Cell transfection, luciferase assay, and gene knockdown

Cells were transfected using FuGENE® HD (Promega, Madison, WI, USA) or Lipofectamine® 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. For the luciferase assay, cells were cultured in 24-well plates and co-transfected with 100 ng of firefly luciferase reporter (IFN- β -Luc) and 10 ng of the constitutive *Renilla luciferase* reporter pRL-TK. Luciferase activity was measured at 24 h post transfection (hpt). Lentiviral shRNA for targeting endogenous DDX21

(5'-CCCATATCTGAAGAACTATT-5') were purchased from Gene Pharma

(Shanghai, China). To generate DDX21 stable knockdown cell line, HeLa cells were

infected with lentiviral shDDX21 and selected by puromycin as described previously

(34).

Immunofluorescence (IF) assay

HeLa cells were washed in PBS, fixed in 4% neutral formaldehyde, and then

permeabilized with 0.5% Triton X-100 in Tris-buffered saline with Tween 20 (TBST)

for 10 min. After blocking in TBST with 3% bovine serum albumin, the cells were

incubated with primary antibody for 1 h at 37°C. The cells were washed three times

with TBST and incubated with secondary antibody. Subsequently, they were washed

and incubated with another primary antibody, followed by incubation with secondary

antibody. Next, the cells were washed again and incubated with 0.5 µg/mL DAPI. The

coverslips were washed and visualized using an LSM 880 Zeiss confocal microscope

(Carl Zeiss, Jena, Germany).

Nucleocytoplasmic separation assay

Nuclear extractions were prepared using NE-PER nuclear and cytoplasmic extraction

reagents (78833, ThermoFisher Scientific) according to the manufacturer's

instructions.

Immunoblotting and co-immunoprecipitation

Immunoblotting was performed as described previously (31). Briefly, cells were lysed in cell lysis buffer containing a protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany). The lysates were denatured and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting and quantified using Image J software. For co-immunoprecipitation, HeLa cells were transfected with expression vectors for 24 or 36 h and lysed with a cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% NP-40) containing 1 mM PMSF and protease inhibitors (Merck-Millipore). The lysates were centrifuged at 12,000 g for 10 min and precipitated with anti-Flag antibody, in conjunction with Protein G Agarose beads (ThermoFisher Scientific) overnight at 4°C. The beads were washed with lysis buffer four times and eluted with SDS loading buffer by boiling for 10 min and then subjected to immunoblotting.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described previously (35). Briefly, total RNA was extracted, reverse transcribed to cDNA, and subjected to qRT-PCR analysis using Premix Ex Taq reagents (Takara, Dalian, China). Primers were referenced from previous reports (35) (Supplementary Table 1). The relative abundance of mRNAs was calculated using the comparative CT ($\Delta\Delta CT$) method (36). All experiments were carried out in triplicate.

Generation of knockout cells

The CRISPR/Cas9 system was used to generate *ddx21* and *casp6* knockout cells. The guide RNA (gRNA) specific to *ddx21* and *casp6* gene was designed using the online CRISPR design tool (<http://crispr.mit.edu/>). The gRNA oligonucleotides were annealed and cloned into the pGK1.1 (Geneloci, Nanjing, China). HeLa cells were electrotransfected with the plasmid at 550V, 1 pulse. After 24 h of electrotransfection, the supernatants were replaced with 10% FBS DMEM supplemented with 1 µg/ml puromycin (Merck-Millipore) for 24 h. The pool (mixed clones) cells were preliminarily sequenced and validated. The candidate positive clones were then subcloned onto 96-well plates using the limiting dilution method. The genomic region surrounding the CRISPR target site was amplified by PCR using the check primers. The primer sequences for sgRNAs and check primers are listed in Supplementary Table 1. The clones were sequenced to ensure the frameshifting mutation of both alleles of the established cell line. Except for PCR verification, the cells were re-checked by immunoblotting using DDX21 antibody. *Casp3*^{-/-} HeLa cells were purchased from EdiGene Inc. (Beijing, China) and verified by immunoblotting. *Casp3/6* double knockout cells were constructed by transfecting *casp6* sgRNA into *casp3*^{-/-} HeLa cells as described above.

Generation of cells stably expressing WT and mutant DDX21

HEK-293T cells were transfected with pHAGE-WT and -D126A DDX21, together with two package plasmids, pMD2.G (Addgene, plasmid 12259) and

psPAX2 (Addgene, plasmid 12260). The supernatants were collected at 60 h post transfection (hpt), centrifuged at 5000 rpm for 10 min, and filtered. The lentivirus supernatants supplemented with 5 µg/mL polybrene (Sigma-Aldrich) were added into the *ddx21*^{+/-} HeLa cells. The cells were horizontally centrifuged at 1000 rpm for 90 min and incubated at 37° C for 48 h. The supernatants were then replaced with 10% FBS supplemented with blasticidin (Merck-Millipore) for 72 h. The cells were subcloned by limiting dilution and confirmed by immunoblotting. The primer sequences for stable expression are listed in Supplementary Table 1.

Statistical analysis

Data were expressed as means ± standard deviations. Significance was determined with the two-tailed independent Student's *t* test ($p < 0.05$) between two groups. A one-way ANOVA was followed by Tukey's to compare multiple groups (> 2).

Results

DDX21 positively regulates IFN- β signaling pathway

To confirm the role of DDX21 in virus replication as well as its role in anti-viral innate immunity, DDX21 was knocked down followed by VSV infection. The results showed that the knockdown of DDX21 significantly reduced the expression of VSV G protein at 12, 18, and 24 h post infection (hpi) (Fig. 1A). Consistently, the virus titers were significantly impaired after DDX21 knockdown at 6, 12, and 18 hpi (Fig. 1B). To study the role of DDX21 in innate immunity, the expression levels of IFN- β and downstream interferon-stimulate gene (ISG), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), were evaluated. The results showed that the knockdown of DDX21 inhibited the mRNA levels of IFN- β (Fig. 1C) and IFIT-1 (Fig. 1D). Consistently, phosphorylation of TBK1 was significantly inhibited at 6, 12 and 18 hpi. Similar results were observed in DDX21 knockdown cells after treatment of poly (I:C), confirmed the positive regulation of IFN- β pathway by DDX21 (Fig. 1E-G). For further confirmation, we generated DDX21 knockout cells. Unfortunately, after three rounds of screening, only one (1/225) heterozygous clone (*ddx21*^{+/-}) was identified by sequencing (Fig. S1A). Western blotting showed that compared with WT cells, DDX21 expression was significantly inhibited in *ddx21*^{+/-} cells (Fig. S1B). The regulation of virus titers and IFN- β pathway was in accord with the knockdown results (Fig. S1C-G). However, using the overexpression model, we observed that transfection of exogenous DDX21 only increased VSV titers at 12 hpi (Fig. 1I), and

the synthesis of virus G protein was unchanged at all time points pi (Fig. 1H).
Correspondingly, DDX21 overexpression had no effect on IFN- β production or the
mRNA levels of ISGs (Fig. 1J–K). Collectively, these results suggested that DDX21
positively regulates IFN- β signaling pathway. However, the discrepancy between the
knockdown and overexpression models raised the question of whether, in addition to
DDX21 expression, the modification of DDX21 plays a role in anti-viral innate
immunity.

The cleavage of DDX21 by virus infection and treatment with RNA/DNA ligands

Interestingly, in both the knockdown and overexpression experiments, we observed
the apparent cleavage of DDX21 in the course of VSV infection (Fig. 1A and H, Fig.
S1C). To further confirm the cleavage of DDX21 by virus infection, cells were
infected by two RNA viruses, VSV and NDV, and one DNA virus, HSV-1,
respectively, followed by DDX21 detection. As expected, VSV and NDV apparently
cleaved DDX21 at 12, 18, and 24 hpi. The full-length DDX21 almost disappeared at
18 and 24 hpi post VSV and NDV infection (Fig. 2A and B). In comparison, HSV-1
only slightly cleaved DDX21, even at the late stage of infection, and the amount of
full-length DDX21 was not significantly decreased (Fig. 2C). Statistically, at 18 and
24 hpi, the ratio of DDX21/cleaved DDX21/ β -actin was 33-564 in VSV- and
NDV-infected cells, compared with 2.4-2.6 in HSV-1-infected cells (Fig. 2D-F).
A549, Huh7, and THP-1 cells were then utilized to test whether virus-triggered
DDX21 cleavage was cell type-dependent. The results showed that DDX21 was

cleaved in A549, Huh7, or THP-1 cells upon VSV or NDV infection (Fig. S2A-D).

Additionally, the cleavage of DDX21 was observed in cells infected with SeV,

another RNA virus (Fig. S2E).

Given that DDX21 was cleaved upon RNA and DNA virus infection, and as DDX21 belongs to the RNA helicase family that can bind various RNAs (22), we propose that this cleavage is triggered by virus nucleotides. Two RNA ligands, poly (I:C) and 3p-hpRNA, and two DNA ligands, poly (G:C) and HSV-60, were used to evaluate their role in DDX21 cleavage. As expected, both RNA and DNA ligands cleaved DDX21 (Fig. 2G and H), and higher cleavage efficiency was observed upon treatment with RNA ligands, compared with that of DNA ligands (Fig. 2I and J), which is in accordance with the results of virus infection. There are two transcript variants of DDX21, isoform 1 (X1), which encodes the full-length of DDX21, and isoform 2 (X2), with a shorter N-terminus (Δ 1-86) compared to X1. Here, we showed that both exogenously expressed DDX21 X1 and X2 were cleaved upon VSV, NDV, and HSV-1 infection (Fig. 2H-J). Collectively, these results clearly demonstrated that DDX21 was cleaved by virus infection and treatment with RNA/DNA ligands.

DDX21 was cleaved in a caspase-dependent manner

To further confirm whether DDX21 was cleaved or degraded upon virus infection and RNA/DNA ligand treatment, cells were treated with caspase inhibitor z-VAD-FMK, neddylation inhibitor MLN4924, proteasome inhibitor MG-132, and autophagy inhibitors wortmannin and CQ, respectively, followed by virus or treatment with

ligand. The results showed that DDX21 cleavage was completely inhibited after treatment with the caspase inhibitor z-VAD-FMK in cells infected with VSV, NDV, and HSV-1 (Fig. 3A–D, lane 4) or treated with RNA ligand poly (I:C) (Fig. 3D, lane 4). Notably, MG132 and CQ also seem to inhibit the cleavage of DDX21, especially in VSV- and NDV-infected cells (Fig. 3A and B, lanes 6 and 7). However, the expression of viral protein was also inhibited after treatment with these two drugs, indicating that the inhibition of DDX21 cleavage may be due to their inhibition of virus replication. In comparison, z-VAD-FMK had no effect on virus replication. These results indicated that DDX21 was cleaved in a caspase-dependent manner.

Caspase-3/6 cleaved DDX21 at D¹²⁶ in response to VSV infection

DDX21 is characterized by several known domains, Q motif (amino acid (aa) 186-214), helicase ATP-binding domain (aa 217-396), and helicase C-terminal domain (aa 429-573) (Fig. 4A). To characterize the cleavage sites of DDX21, based on the identified domains, several deletion mutants (Δ 1-216, Δ 217-395, Δ 396-573, Δ 574-784) were generated to test the critical domain involved in DDX21 cleavage. As shown in Fig. 4B, deletion of 1-216 abrogated the cleavage of DDX21. Notably, unlike endogenous DDX21 expression, exogenous expression of DDX21 truncates alone is able to induce cleavage to some extent (Fig. 4B, left panel). Similar results could be observed in the overexpression of wild-type (WT) DDX21 (Fig. 2H-J). Combined with the results showing that aa 1-216 is critical for DDX21 (Fig. 4B) and aa 1-86 is not required for DDX21 cleavage (Fig. 2H-J), the cleavage sites are within

aa 87-216 of DDX21. The caspase cleavage sites were then predicted by CaspDB (<http://caspdb.sanfordburnham.org>). The results showed that all three aspartates (Asp, D), D87, D126, D160, are the putative caspase cleavage sites (Fig. 4C). Therefore, DDX21 with single, double, and triple mutations of these three Asp sites were generated, respectively, followed by transfection and virus infection. The results showed that D126A, but not D87A or D160A, is sufficient and necessary for DDX21 cleavage (Fig. 4D, lanes 4, 6, 8 and 9). The truncates of DDX21 1-125 and 127-784, together with WT DDX21, were transfected into cells to further confirm that the cleavage was mediated by D126. As expected, WT, but not 1-125 and 127-784 DDX21, was cleaved upon virus infection (Fig. 4E). It should be noted that based on the molecular weight of DDX21, we proposed that the “cDDX21” we observed in endogenous cleavage (~73kD) was DDX21 127-784, while the relatively small cleaved DDX21 (~14kD) was degraded upon virus infection (Fig. 4E). Previous reports showed that the cleavage sites specific for caspases had a general motif (37). The motif for DDX21 cleavage is Glu-Ile-Asp (E-I-D), which is the putative substrate for caspase-3 or caspase-6 (Fig. 4F). *casp3*^{-/-} and *casp6*^{-/-} cells were generated to verify their role in DDX21 cleavage. As expected, knockout of *casp3* and especially *casp6* significantly inhibited the cleavage of DDX21 (Fig. 4G-H). DDX21 cleavage was almost blocked in *casp3/6* double knockout cell lines (Fig. 4I). These results indicated that virus infection cleaved DDX21 at D126 via caspase-3/6.

DDX21 was cleaved and translocated from the nucleus to the cytoplasm in response to virus infection

DDX21 was identified to be a nucleolar helicase that is required for pre-rRNA processing (22, 23, 38). Other reports showed that DDX21 is localized with DDX1, DDX26, and TRIF to sense dsRNA in the cytosol (20). To study whether DDX21 localization was affected by virus infection, cells were infected with VSV and HSV-1, followed by IFA test. As expected, endogenous DDX21 was predominantly localized in the nucleolus in mock-infected cells (Fig. 5A and C, upper panels). DDX21 was translocated from the nucleolus to nucleoplasm and cytoplasm after VSV and HSV-1 infection (Fig. 5A and C, middle and lower panel). Statistically, at 12 and 24 hpi, cells with cytoplasmic DDX21 accounted for 23% and 68% of all cells for VSV (Fig. 5B) and 24% and 49% of all cells for HSV-1 (Fig. 5D), respectively, indicating that VSV could induce DDX21 translocation more efficiently than HSV-1. Next, we evaluated whether the cleavage of DDX21 affected its localization. WT, D126A, 1-125, and 127-784 DDX21 (Fig. 5E), together with empty vector, were transfected into cells followed by VSV infection. The results showed that in mock-infected cells, WT, D126A, and 127-784 DDX21 predominantly localized in the nucleolus (Fig. 5F, left panel). WT and 127-784, but not D126A DDX21, efficiently translocated from the nucleolus to nucleoplasm and cytoplasm after VSV infection (Fig. 5F and G), indicating that blockage of DDX21 cleavage inhibited its translocation. Moreover, the statistical analysis results showed the most efficient translocation of DDX21 cleaved

form (cDDX21, 126-784), further indicating DDX21 cleavage promotes its translocation from nucleus to cytoplasm. Interestingly, 1-125 DDX21 was diffusely localized in the nucleus and cytoplasm in both mock- and VSV-infected cells (Fig. 5F and G). Collectively, these results demonstrated that the virus induced the translocation of DDX21, which is affected by DDX21 cleavage.

DDX21 cleavage led to the inhibition of the IFN- β signaling pathway

Given that the blockage of DDX21 inhibits its translocation, we next aimed to explore the effect of DDX21 cleavage in the regulation of innate immunity. *Ddx21*^{+/-} cells were transfected with WT and truncated DDX21, followed by the detection of virus replication and IFN- β signaling pathway. Interestingly, transfection of WT and truncated DDX21 did not affect virus replication, evidenced by viral protein expression and titers in the supernatants (Fig. 6A and B). In contrast, although WT DDX21 has no effect, the intact form of DDX21 (D126A) did increase the mRNA levels of IFN- β and ISGs (IFIT-1 and MX1) after VSV infection. More importantly, transfection of cDDX21 significantly inhibited IFN- β signaling pathway (Fig. 6C-E). These results were further confirmed using poly (I:C) as the stimulator (Fig. 6F-H). To identify whether DDX21 cleavage regulates IFN- β signaling pathway in a time-dependent manner, *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 infected by VSV or treated with poly (I:C) and collected at different time points. DDX21 was cleaved in WT but not D126A DDX21-expressed cells. However, no difference was observed in viral protein synthesis between WT and D126A

DDX21-expressed cells (Fig. 6I). More importantly, blockage the cleavage (D126A) significantly increased the IFN- β promoter activity (Fig. 6K and O), mRNA levels of IFN- β and ISGs (IFIT-1 and MX1) (Fig. 6L–N, P–R) and IFN- β production in the supernatants (Fig. 6S) especially at the late stage of virus infection and poly (I:C) treatment. These results clearly indicated that DDX21 cleavage inhibits the IFN- β signaling pathway.

Cleavage of DDX21 inhibited the formation of DDX1-DDX21-DHX36 complex

Given that DDX21 was reported to act as the scaffold protein in the complex of three dsRNA-sensing helicases (DDX1-DDX21-DHX36) (20, 39) (Fig. 7D), we hypothesized that DDX21 cleavage attenuates the inhibition of the IFN- β signaling pathway by inhibition of the formation of DDX1-DDX21-DHX36 complex. Since DDX21 was reported to exist as a homodimer (25, 27), the cleavage of DDX21 on its self-interaction was studied first. As shown in Fig. 7A, using full-length DDX21 as the bait, the full-length and 127-784, but not 1-125 DDX21, could be immunoprecipitated (Fig. 7A). In accordance, 127-784 DDX21 was interacted with 127-784 and full-length, but not 1-125 DDX21 (Fig. 7B). In contrast, 1-125 DDX21 was not immunoprecipitated with WT and truncated DDX21 (Fig. 7C). These results indicated that DDX21 interacted with itself through its C-terminal 127-784. Next, we evaluated whether DDX21 cleavage inhibits the formation of DDX1-DDX21-DHX36 complex. The results showed that compared with intact DDX21 (D126A), cDDX21 (127-784) has a lower affinity for both DDX1 and TRIF (Fig. 7E–F). In comparison,

399 D126A, 127-784 and 1-125 DDX21 showed similar binding ability to DHX36 (Fig.
400 7G). Collectively, these results indicated cleavage of DDX21 inhibited the formation
401 of DDX1-DDX21-DHX36 complex and its interaction to downstream adaptor TRIF.

Discussion

Intensive functional and structural research over the years has clearly demonstrated that RLRs selectively bind viral RNA ligands and trigger downstream signaling (40). Several other DExD/H-box helicases have been implicated in anti-viral innate immunity, but the fundamental questions such as the precise regulation mechanism how DExD/H-box helicases regulate innate immunity remain to be elucidated. Here, we demonstrated that DDX21 is cleaved at D¹²⁶ after virus infection and RNA/DNA ligand treatment via the caspase-3/6 pathway, leading to the inhibition of immune responses.

To date, there has been limited research on the cleavage of RNA helicases. Human RNA helicase A, a nuclear helicase that unwinds dsRNA, dsDNA, and RNA:DNA duplexes, was reported to be cleaved by caspase-3 during apoptosis (41). Another study also showed that the CARD-containing helicase (Helicard) is cleaved by caspases upon apoptotic stimuli (42). However, no studies have shown that the cleavage of DNA/RNA helicases is involved in innate immunity and virus infection. Our data showed the obvious cleavage of DDX21 in response to virus infection and treatment with ligands. The fact that three RNA viruses and one DNA virus, together with two RNA and two DNA ligands, trigger varying degrees of DDX21 cleavage leads us to speculate that the ability of DDX21 cleavage may be shared by most viruses.

The caspase family of cysteine proteases is involved in apoptosis and innate immune signaling (43). In terms of the innate immunity pathway, caspase-1 is one of the most well-studied caspases, which is able to cleave proIL-1 β and proIL-18 and triggers inflammasomes (44). It has also been reported that caspases are involved in the RLR-mediated type I IFN response. For instance, caspase-12 positively modulates the IFN- β signaling pathway by regulating E3 ubiquitin ligase TRIM25-mediated ubiquitination of RIG-I (45). Activated caspase-3/7/9 suppress mitochondrial DNA-induced stimulator of interferon genes protein (STING)-mediated type I IFN production (46). Here, we showed that the cleavage of DDX21 was completely recovered in the presence of caspase inhibitor z-VAD-FMK (Fig. 3), and the cleavage was probably mediated by caspase-3/6 (Fig. 4). It should be noted that knockout of *casp3* and *casp6* did not completely blocked DDX21 cleavage, indicating that the involvement of other caspases could not be completely excluded. A recent study revealed that caspase-6 cleaves IL-1R-associated kinase-M (IRAK-M) and reduces I κ B α degradation, thereby increasing TNF- α production (47). The function of caspase-6 in the type I IFN pathway has not been reported. Our study indicated that caspase-3/6 cleaves DDX21 and thus likely regulates the IFN- β signaling pathway.

In a resting state, DDX21, together with its binding partners, c-Jun, WDR46, SIRT7, etc., is localized in the cell nucleolus (23, 38, 48). The nucleolar localization of DDX21 is necessary for its pre-rRNA processing and RNA unwinding (23, 48). Studies have also suggested that DDX21 is involved in innate immunity in the

cytoplasm. For example, the DDX1-DDX21-DHX36-TRIF complex may translocate to the mitochondria upon poly(I:C) stimulation (20). The infection of A549 cells with the dengue virus causes DDX21 to partially relocate from the nucleus to the cytoplasm (29). Here, we confirmed that DDX21 localized in the cell nucleolus in mock-infected cells. Virus infection effectively triggered DDX21 translocation from the nucleolus to the cytoplasm. In resting cells, WT, D126A, and 127-784 DDX21 showed nucleolar localization. It is interesting that 1-125 DDX21 diffusely localized in the nucleus and the cytoplasm. Previous reports showed that the 731–740 at the C-terminus of DDX21 interacted with c-Jun, and depletion of c-Jun promotes DDX21 translocation from the nucleolus to the nucleoplasm (23). Therefore, it is possible that the deletion of the C-terminus of DDX21 abolishes the interaction with its binding partner and thus alters its nucleolar localization. Our results that (1) blockage of DDX21 cleavage inhibits DDX21 translocation, (2) the most efficient translocation of cDDX21 upon virus infection, indicated that cleavage of DDX21 promotes its translocation from nucleus to cytoplasm.

Various DDX and DHX helicases play important roles in maintaining the stability of the cell genome (22, 48, 49). To date, the knockout of *ddx21* has never been reported in cells or mice. Studies on other DDXs have also shown that certain DDXs are critical for mouse growth, and knockout of the *ddx* gene results in early embryonic lethality (50, 51). Therefore, DDX21 may also be critical for cell and mouse survival. Nevertheless, DDX21 expression was significantly inhibited in

ddx21^{+/-} cells. Previous studies and our results demonstrated that depletion of DDX21 significantly inhibited type I IFN production (20), suggesting that DDX21 positively regulate innate immunity. Interestingly, depletion of DDX21 also impaired virus replication. Considering DDX21 is a multifunctional protein which also plays a important role in maintaining the stability of the cell genome (27, 48, 52), it may also influence virus replication in other ways except for innate immunity. Nevertheless, it is undoubtful that DDX21 *per se* plays a positive role in innate immunity. Most importantly, cleavage of DDX21 inhibits innate immunity, but not affects virus infection, which lead us to speculate the cleavage of DDX21 was driven by host for a late counter-regulatory effect to temper immune responses.

Although type I IFN is widely reported to play an essential role against viral infection, the aberrant production of cytokines leads to unexpected pathological consequences in a variety of autoimmune diseases (53, 54). Therefore, the balance between these key pathways is essential for immune homeostasis. Indeed, DNA sensor cGAS has been reported as a key driver of lethal autoimmune disease in the Trex1-deficient mouse model of Aicardi–Goutieres Syndrome (AGS) (55). The importance of excess RLR-dependent signaling, which leads to an IFN signature in the pathogenesis of many autoimmune diseases, such as AGS and systemic lupus erythematosus, has also been clarified (56). These reports highlight the importance of fine-tuning the regulation of the type I IFN signaling pathway. Here, we provided several lines of evidence to demonstrate that host promotes DDX21 cleavage to

temper immune responses. (i) the cleavage of DDX21 was observed at the late stage of infection; (ii) the cleavage was a universal phenomenon not only for virus but also for RNA and DNA mimics. (iii) blockage of DDX21 (D126A) cleavages increase IFN production and cleaved DDX21 (127-784aa) inhibits IFN production. (iv) cleavage of DDX21 did not affect virus replication at the late stage of infection. (v) cleavage of DDX21 inhibited the formation of DDX1-DDX21-DHX36 complex. Collectively, from our original and additional data, we inferred that host promotes DDX21 cleavage via caspase3/6 to suppress DDX1-DDX21-DHX36 complex formation for a late counter-regulatory effect to temper immune responses (Fig. 8). An improved understanding of these processes could shed light on the causes of infectious disease and, plausibly, immune disorders involving excessive inflammatory immune activities.

498 **Acknowledgements**

499 We thank Dr. Takashi Fujita (Kyoto University) for providing plasmid
500 IFN- β -Luc. We thank Dr. Yasushi Kawaguchi (university of Tokyo, Japan) for
501 providing HSV-1. We thank Dr. Jianchao Wei (Shanghai Veterinary Research
502 Institute, China) for providing VSV. We thank Quan Zhang (Yangzhou University,
503 China) for providing Sendai virus (SeV).

504 **Funding information**

505 This work was funded by grants 32030108 (to C. D) and 31872453 (to Y. S) from the
506 National Natural Science Foundation of China and by grant 2018YFD0500100 from
507 the National Key Research and Development Program of China to C. D. The funders
508 had no role in study design, data collection and interpretation, or the decision to
509 submit the work for publication.

510

Reference

1. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140:805-20.
2. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-8.
3. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 169:6668-72.
4. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441:101-5.
5. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5:730-7.
6. Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 19:727-40.
7. Seth RB, Sun L, Ea CK, Chen ZJ. 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 122:669-82.
8. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437:1167-72.
9. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6:981-8.
10. Sun Q, Sun L, Liu HH, Chen X, Seth RB, Forman J, Chen ZJ. 2006. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* 24:633-42.
11. Patel SS, Donmez I. 2006. Mechanisms of helicases. *J Biol Chem* 281:18265-8.
12. Umate P, Tuteja N, Tuteja R. 2011. Genome-wide comprehensive analysis of human helicases. *Commun Integr Biol* 4:118-37.
13. Singleton MR, Dillingham MS, Wigley DB. 2007. Structure and mechanism of helicases and nucleic acid translocases. *Annu Rev Biochem* 76:23-50.
14. Steimer L, Klostermeier D. 2012. RNA helicases in infection and disease. *RNA Biol* 9:751-71.

- 552 15. Fairman-Williams ME, Guenther UP, Jankowsky E. 2010. SF1 and SF2
553 helicases: family matters. *Curr Opin Struct Biol* 20:313-24.
- 554 16. Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, Kato N. 2007.
555 DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA
556 replication. *J Virol* 81:13922-6.
- 557 17. Miyashita M, Oshiumi H, Matsumoto M, Seya T. 2011. DDX60, a DEXD/H
558 box helicase, is a novel antiviral factor promoting RIG-I-like
559 receptor-mediated signaling. *Mol Cell Biol* 31:3802-19.
- 560 18. Oshiumi H, Ikeda M, Matsumoto M, Watanabe A, Takeuchi O, Akira S, Kato
561 N, Shimotohno K, Seya T. 2010. Hepatitis C virus core protein abrogates the
562 DDX3 function that enhances IPS-1-mediated IFN-beta induction. *PLoS One*
563 5:e14258.
- 564 19. Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, Pfeffer S,
565 Landthaler M, Landgraf P, Kan S, Lindenbach BD, Chien M, Weir DB, Russo
566 JJ, Ju J, Brownstein MJ, Sheridan R, Sander C, Zavolan M, Tuschl T, Rice
567 CM. 2007. Cellular cofactors affecting hepatitis C virus infection and
568 replication. *Proc Natl Acad Sci U S A* 104:12884-9.
- 569 20. Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, Qin J, Cheng
570 G, Liu YJ. 2011. DDX1, DDX21, and DHX36 helicases form a complex with
571 the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity*
572 34:866-78.
- 573 21. Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ. 2011. The helicase DDX41
574 senses intracellular DNA mediated by the adaptor STING in dendritic cells.
575 *Nat Immunol* 12:959-65.
- 576 22. Calo E, Flynn RA, Martin L, Spitale RC, Chang HY, Wysocka J. 2015. RNA
577 helicase DDX21 coordinates transcription and ribosomal RNA processing.
578 *Nature* 518:249-53.
- 579 23. Holmstrom TH, Mialon A, Kallio M, Nymalm Y, Mannermaa L, Holm T,
580 Johansson H, Black E, Gillespie D, Salminen TA, Langel U, Valdez BC,
581 Westermarck J. 2008. c-Jun supports ribosomal RNA processing and nucleolar
582 localization of RNA helicase DDX21. *J Biol Chem* 283:7046-53.
- 583 24. Henning D, So RB, Jin R, Lau LF, Valdez BC. 2003. Silencing of RNA
584 helicase II/Gualpha inhibits mammalian ribosomal RNA production. *J Biol*
585 *Chem* 278:52307-14.
- 586 25. Flores-Rozas H, Hurwitz J. 1993. Characterization of a new RNA helicase
587 from nuclear extracts of HeLa cells which translocates in the 5' to 3' direction.
588 *J Biol Chem* 268:21372-83.
- 589 26. McRae EKS, Davidson DE, Dupas SJ, McKenna SA. 2018. Insights into the
590 RNA quadruplex binding specificity of DDX21. *Biochim Biophys Acta Gen*
591 *Subj* 1862:1973-1979.

- 592 27. McRae EKS, Booy EP, Moya-Torres A, Ezzati P, Stetefeld J, McKenna SA.
593 2017. Human DDX21 binds and unwinds RNA guanine quadruplexes. *Nucleic*
594 *Acids Res* 45:6656-6668.
- 595 28. Valdez BC, Henning D, Perumal K, Busch H. 1997. RNA-unwinding and
596 RNA-folding activities of RNA helicase II/Gu--two activities in separate
597 domains of the same protein. *Eur J Biochem* 250:800-7.
- 598 29. Dong Y, Ye W, Yang J, Han P, Wang Y, Ye C, Weng D, Zhang F, Xu Z, Lei
599 Y. 2016. DDX21 translocates from nucleus to cytoplasm and stimulates the
600 innate immune response due to dengue virus infection. *Biochem Biophys Res*
601 *Commun* 473:648-53.
- 602 30. Chen G, Liu CH, Zhou L, Krug RM. 2014. Cellular DDX21 RNA helicase
603 inhibits influenza A virus replication but is counteracted by the viral NS1
604 protein. *Cell Host Microbe* 15:484-93.
- 605 31. Sun Y, Yu S, Ding N, Meng C, Meng S, Zhang S, Zhan Y, Qiu X, Tan L,
606 Chen H, Song C, Ding C. 2014. Autophagy benefits the replication of
607 Newcastle disease virus in chicken cells and tissues. *J Virol* 88:525-37.
- 608 32. Li X, Qiu Y, Shen Y, Ding C, Liu P, Zhou J, Ma Z. 2008. Splicing together
609 different regions of a gene by modified polymerase chain reaction-based
610 site-directed mutagenesis. *Anal Biochem* 373:398-400.
- 611 33. Zheng L, Baumann U, Reymond JL. 2004. An efficient one-step site-directed
612 and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32:e115.
- 613 34. Xu W, Zhang Z, Zou K, Cheng Y, Yang M, Chen H, Wang H, Zhao J, Chen P,
614 He L, Chen X, Geng L, Gong S. 2017. MiR-1 suppresses tumor cell
615 proliferation in colorectal cancer by inhibition of Smad3-mediated tumor
616 glycolysis. *Cell Death Dis* 8:e2761.
- 617 35. Sun Y, Ding N, Ding SS, Yu S, Meng C, Chen H, Qiu X, Zhang S, Yu Y,
618 Zhan Y, Ding C. 2013. Goose RIG-I functions in innate immunity against
619 Newcastle disease virus infections. *Mol Immunol* 53:321-7.
- 620 36. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the
621 comparative C(T) method. *Nat Protoc* 3:1101-8.
- 622 37. McStay GP, Salvesen GS, Green DR. 2008. Overlapping cleavage motif
623 selectivity of caspases: implications for analysis of apoptotic pathways. *Cell*
624 *Death Differ* 15:322-31.
- 625 38. Hirai Y, Louvet E, Oda T, Kumeta M, Watanabe Y, Horigome T, Takeyasu K.
626 2013. Nucleolar scaffold protein, WDR46, determines the granular
627 compartmental localization of nucleolin and DDX21. *Genes Cells* 18:780-97.
- 628 39. Gantier MP, Williams BR. 2011. Making sense of viral RNA sensing. *Mol*
629 *Ther* 19:1578-81.
- 630 40. Yoneyama M, Onomoto K, Jogi M, Akaboshi T, Fujita T. 2015. Viral RNA
631 detection by RIG-I-like receptors. *Curr Opin Immunol* 32:48-53.

- 632 41. Takeda Y, Caudell P, Grady G, Wang G, Suwa A, Sharp GC, Dynan WS,
633 Hardin JA. 1999. Human RNA helicase A is a lupus autoantigen that is
634 cleaved during apoptosis. *J Immunol* 163:6269-74.
- 635 42. Kovacsovics M, Martinon F, Micheau O, Bodmer JL, Hofmann K, Tschopp J.
636 2002. Overexpression of Helicard, a CARD-containing helicase cleaved
637 during apoptosis, accelerates DNA degradation. *Curr Biol* 12:838-43.
- 638 43. Creagh EM. 2014. Caspase crosstalk: integration of apoptotic and innate
639 immune signalling pathways. *Trends Immunol* 35:631-640.
- 640 44. Latz E, Xiao TS, Stutz A. 2013. Activation and regulation of the
641 inflammasomes. *Nat Rev Immunol* 13:397-411.
- 642 45. Wang P, Arjona A, Zhang Y, Sultana H, Dai J, Yang L, LeBlanc PM, Doiron
643 K, Saleh M, Fikrig E. 2010. Caspase-12 controls West Nile virus infection via
644 the viral RNA receptor RIG-I. *Nat Immunol* 11:912-9.
- 645 46. White MJ, McArthur K, Metcalf D, Lane RM, Cambier JC, Herold MJ, van
646 Delft MF, Bedoui S, Lessene G, Ritchie ME, Huang DC, Kile BT. 2014.
647 Apoptotic caspases suppress mtDNA-induced STING-mediated type I IFN
648 production. *Cell* 159:1549-62.
- 649 47. Kobayashi H, Nolan A, Naveed B, Hoshino Y, Segal LN, Fujita Y, Rom WN,
650 Weiden MD. 2011. Neutrophils activate alveolar macrophages by producing
651 caspase-6-mediated cleavage of IL-1 receptor-associated kinase-M. *J Immunol*
652 186:403-10.
- 653 48. Song C, Hotz-Wagenblatt A, Voit R, Grummt I. 2017. SIRT7 and the
654 DEAD-box helicase DDX21 cooperate to resolve genomic R loops and
655 safeguard genome stability. *Genes Dev* 31:1370-1381.
- 656 49. Hodroj D, Serhal K, Maiorano D. 2017. Ddx19 links mRNA nuclear export
657 with progression of transcription and replication and suppresses genomic
658 instability upon DNA damage in proliferating cells. *Nucleus* 8:489-495.
- 659 50. Ma Z, Moore R, Xu X, Barber GN. 2013. DDX24 negatively regulates
660 cytosolic RNA-mediated innate immune signaling. *PLoS Pathog* 9:e1003721.
- 661 51. Janknecht R. 2010. Multi-talented DEAD-box proteins and potential tumor
662 promoters: p68 RNA helicase (DDX5) and its paralog, p72 RNA helicase
663 (DDX17). *Am J Transl Res* 2:223-34.
- 664 52. Xing YH, Yao RW, Zhang Y, Guo CJ, Jiang S, Xu G, Dong R, Yang L, Chen
665 LL. 2017. SLERT Regulates DDX21 Rings Associated with Pol I
666 Transcription. *Cell* 169:664-678 e16.
- 667 53. Nallar SC, Kalvakolanu DV. 2014. Interferons, signal transduction pathways,
668 and the central nervous system. *J Interferon Cytokine Res* 34:559-76.
- 669 54. Crow MK. 2014. Advances in understanding the role of type I interferons in
670 systemic lupus erythematosus. *Curr Opin Rheumatol* 26:467-74.
- 671 55. Gray EE, Treuting PM, Woodward JJ, Stetson DB. 2015. Cutting Edge: cGAS
672 Is Required for Lethal Autoimmune Disease in the Trex1-Deficient Mouse
673 Model of Aicardi-Goutieres Syndrome. *J Immunol* 195:1939-43.

- 674 56. Kato H, Fujita T. 2015. RIG-I-like receptors and autoimmune diseases. Curr
675 Opin Immunol 37:40-5.

676

Figure Legends

Figure 1. DDX21 positively regulates IFN pathway

(A) HeLa cells with stable knockdown of DDX21 and control cells were mock treated or infected with VSV at an MOI of 1, respectively. Cells were harvested at 6, 12, 18, and 24 hpi, and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, anti-p-TBK1, anti-TBK1 or anti- β -actin antibody.

(B) Extracellular virus yields in DDX21 knockdown and control cells.

(C-D) Virus infection experiments were performed as in A. Cells were harvested and detected using qRT-PCR with IFN- β (C) and IFIT-1(D) primers, respectively.

(E) HeLa cells with stable knockdown of DDX21 and control cells were mock treated or transfected with poly (I:C) (20 μ g/mL), respectively. Cells were harvested at 6, 12 and 18 hpt and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, anti-p-TBK1, anti-TBK1 or anti- β -actin antibody.

(F-G) Poly (I:C) treatment experiments were performed as in E. Cells were harvested and detected using qRT-PCR with IFN- β (F) and IFIT-1(G) primers, respectively.

(H) HeLa cells were transfected with either empty vector or Flag-DDX21. At 24 h after transfection, cells were mock treated or infected with VSV at an MOI of 1. Cells were harvested at 6, 12, 18, and 24 hpi, and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, or anti- β -actin antibody.

(I) Extracellular virus yields in empty vector or Flag-DDX21 transfected group.

(J-K) Virus infection experiments were performed as in G. Cells were harvested and detected using qRT-PCR with IFN- β (J) and IFIT-1(K) primers, respectively. Data are presented as means from three independent experiments. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 2. Virus infection or treatment with RNA/DNA ligands leads to the cleavage of DDX21

(A-C) HeLa cells were mock treated or infected with VSV (A), NDV (B) or HSV-1 (C), respectively, at an MOI of 1. Cells were harvested at 6, 12, 18, and 24 hpi, and detected using immunoblot analysis with anti-DDX21, anti- β -actin or anti-viral-protein (VSV-G, NDV-NP, HSV-1-gD) antibody.

(D-F) Representative results are shown with graphs representing the intensity band ratio of cleaved DDX21 (cDDX21)/DDX21/ β -actin normalized to the control condition for VSV (D), NDV (E) and HSV-1 (F) infection group. Data are presented as means from three independent experiments. ***, $P < 0.001$.

(G-H) HeLa cells were transfected with RNA ligands (poly (I:C) or 3p-hpRNA, G) or DNA ligands (poly (G:C) or HSV-60, H), respectively. At 18 hours post transfection (hpt), cells were harvested and detected using immunoblot analysis with anti-DDX21 or anti- β -actin antibody.

(I-J) Representative results are shown with graphs representing the intensity band ratio of cleaved DDX21 (cDDX21)/DDX21/ β -actin normalized to the control condition for RNA (I) and DNA (J) ligand treatments group.

(K) Schematic representation of two transcript isoforms of DDX21.

(H-J) HeLa cells were transfected with either empty vector, Flag-DDX21-X1, Flag-DDX21-X2. At 24 h after transfection, cells were mock treated or infected with VSV (H), NDV (I) or HSV-1 (J), respectively, at an MOI of 1. Cells were harvested at 18 hpi, and detected using immunoblot analysis with anti-DDX21, anti- β -actin or anti-viral-protein (VSV-G, NDV-NP, HSV-1-gD) antibody.

Figure 3. The caspase-dependent cleavage of DDX21 triggered by virus infection or treatments of RNA ligands

(A-C) HeLa cells were mock infected or infected with VSV(A), NDV (B) or HSV-1 (C) at an MOI of 1 and maintained in the presence of DMSO control, z-VAD-FMK (VAD), MLN4924 (MLN), MG132 (MG), CQ or wortmannin (Wort) for 18 h. Cells were harvested and detected using immunoblot analysis with anti-DDX21, anti- β -actin or anti-viral-protein (VSV-G, HSV-1-gD, NDV-NP) antibody.

(D) HeLa cells were mock treated or transfected with RNA ligands (poly (I:C) and maintained in the presence of DMSO control, z-VAD-FMK, MLN4924, MG132, CQ or wortmannin for 18 h. Cells were harvested and detected using immunoblot analysis with anti-DDX21 or anti- β -actin antibody.

Figure 4. DDX21 was cleaved at D¹²⁶ by caspase-3/6 in response to VSV infection

(A) Schematic representation of WT DDX21 and its deletion mutants.

(B) HeLa cells were transfected with Flag tagged DDX21 truncates (Δ 1-216, Δ 217-395, Δ 396-573, Δ 574-784). At 24 h after transfection, cells were mock treated or infected with VSV at an MOI of 1. Cells were harvested at 18 hpi, and detected using immunoblot analysis with anti-Flag, anti-VSV-G, or anti- β -actin antibody.

(C) The prediction results of caspase cleavage sites for DDX21 based on CaspDB (<http://caspdb.sanfordburnham.org>). The red frame indicates aa 87-216 of DDX21. The red asterisks (*) indicate the putative caspase cleavage sites.

(D) HeLa cells were transfected with either empty vector or Flag tagged WT and mutants of DDX21 (D87A, D126A, 160A, D87A/D126A, D87A/160A, D126A/D160A, D81A/D126A/160A). At 24 h after transfection, cells were mock treated or infected with VSV at an MOI of 1. Cells were harvested at 18 hpi, and detected using immunoblot analysis with anti-Flag, anti-VSV-G, or anti- β -actin antibody.

(E) HeLa cells were transfected with either empty vector or Flag tagged WT and truncates of DDX21 (1-125, 127-784). At 24 h after transfection, cells were mock treated or infected with VSV at an MOI of 1. Cells were harvested at 18 hpi, and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, or anti- β -actin antibody.

(F) Schematic representation of amino acids around cleavage site. The red frame indicates the general motif for caspase cleavage.

(G-I) WT and *casp3* (G), *casp6* (H) knockout and *casp3/6* double-knockout (I)

HeLa cells were mock treated or infected with VSV at an MOI of 1, respectively.

Cells were harvested at 18 hpi, and detected using immunoblot analysis with

anti-DDX21, anti-caspase-3/6, anti-VSV-G, or anti- β -actin antibody.

Representative results are shown with graphs representing the intensity band ratio

of cleaved DDX21 (cDDX21)/DDX21/ β -actin normalized to the control

condition.

Figure 5. Virus infection triggers the translocation of DDX21 from nucleus to cytoplasm which is dependent on its cleavage

(A, C) HeLa cells were mock treated or infected with VSV (A) or HSV-1 (C) at

an MOI of 1. At 6 and 12 hpi, cells were fixed, and processed for IF using

anti-DDX21 or anti-viral-protein (VSV-G, HSV-1-gD) antibody. Nuclei were

stained with 1 μ g/mL of DAPI.

(B, D) The relative percentages of cells with nuclear and cytoplasmic DDX21

staining upon VSV (B) or HSV-1 (D) infection were quantified. 10 images in 20

high-powered fields (HPFs) were obtained randomly in different fields.

(F) HeLa cells were transfected with either empty vector or Flag tagged WT,

D126A, 1-126 or 127-784 DDX21. At 24 h after transfection, cells were mock

treated or infected with VSV at an MOI of 1. At 12 hpi, cells were fixed, and

processed for IF using anti-Flag or anti-VSV-G antibody. Nuclei were stained

with 1 μ g/mL of DAPI.

(G) The relative percentages of cells with nuclear and cytoplasmic DDX21 staining in cells transfected with either empty vector or Flag tagged WT, D126A, 1-126 or 127-784 DDX21 upon virus infection were quantified. 10 images in 20 high-powered fields (HPFs) were obtained randomly in different fields.

Figure 6. Cleavage of DDX21 impairs IFN- β production

(A) *ddx21*^{+/-} HeLa cells were transfected with either empty vector or Flag tagged WT, D126A, 1-126 or 127-784 DDX21. At 24 h after transfection, cells were mock treated or infected with VSV at an MOI of 1. At 18 hpi, cells were harvested and detected using immunoblot analysis with anti-Flag, anti-VSV or anti- β -actin antibody. The arrow indicates cleaved DDX21.

(B) Extracellular virus yields in WT and truncated DDX21 transfected *ddx21*^{+/-} HeLa cells.

(C-E) Virus infection experiments were performed as in A. Cells were harvested and detected using qRT-PCR with IFN- β (C), IFIT-1(D) and MX1 (E) primers, respectively.

(F-H) *ddx21*^{+/-} HeLa cells were transfected with either empty vector or Flag tagged WT, D126A, 1-126 or 127-784 DDX21. At 24 h after transfection, cells were mock treated or transfected with poly (I:C) (20 μ g/mL), respectively. Cells were harvested at 12 hpt and detected using qRT-PCR with IFN- β (F), IFIT-1 (G), or MX1 (H) primers.

(I) *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 were mock treated or infected with VSV at an MOI of 1, respectively. Cells were harvested at 6, 12, 18 and 24 hpi, and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, or anti-β-actin antibody.

(J) Extracellular virus yields in *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21

(K) *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 were co-transfected with IFN-β-Luc and PRL-TK. At 12 hpt, cells were mock treated or infected with VSV at an MOI of 1, respectively. Cells were harvested at 6, 12 and 18 hpi and assessed for luciferase activity. The results are presented as relative luciferase activity.

(L-N) *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 were mock treated or infected with VSV at an MOI of 1, respectively. Cells were harvested at 6, 12 and 18 hpi and detected using qRT-PCR with IFN-β (L), IFIT-1 (M), or MX1 (N) primers. The results are presented as relative luciferase activity.

(O) *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 were co-transfected with p-125Luc and PRL-TK. At 12 hpt, cells were mock treated or transfected with poly (I:C) (20 μg/mL). Cells were harvested at 6, 12 and 18 hpt and assessed for luciferase activity. The results are presented as relative luciferase activity.

(P-R) *ddx21*^{+/-} cells stably expressing flag-tagged WT and D126A DDX21 were mock treated or transfected with poly (I:C) (20 µg/mL), respectively. Cells were harvested at 6, 12 and 18 hpt and detected using qRT-PCR with IFN-β (P), IFIT-1 (Q), or MX1 (R) primers.

(S) Poly (I:C) treatments were performed as in O. Supernatants were collected for quantization of IFN-β by ELISA.

Data are presented as means from three independent experiments. *, P<0.05, **, P<0.01, ***, P<0.001.

Figure 7. DDX21 cleavage inhibits its interaction with DDX1 and TRIF

(A-C) HeLa cells were transfected with Flag tagged WT (A), 127-784 (B), 1-125 (C) DDX21, respectively, together with HA tagged WT, 1-125 or 127-784 DDX21. At 24 hpt, cells were harvested, immunoprecipitated with anti-Flag antibody, and further detected using immunoblot analysis with anti-HA or anti-Flag antibody. Expression levels of the proteins were analyzed by immunoblot analysis of the lysates with anti-HA or anti-Flag antibody. The asterisk (*) indicates the predicted size of HA-DDX21 (1-125).

(D) The existing model of foreign RNA recognition by DDX1-DDX21-DDX36 complex.

(E-G) HeLa cells were co-transfected with Flag-tagged D126A, 1-125, 127-784 DDX21 or empty vector, and HA-tagged DDX1 (E), HA-tagged TRIF (F) and Myc-tagged DHX36 (G), respectively. At 24 hpt, cells were harvested,

immunoprecipitated with anti-Flag antibody, and further detected using immunoblot analysis with anti-HA or anti-Flag antibody. Expression levels of the proteins were analyzed by immunoblot analysis of the lysates with anti-HA or anti-Flag antibody.

Figure 8. Proposed model for the regulation of innate immunity by DDX21 cleavage during virus infection

The double-edged sword role of DDX21 in regulation of innate immunity upon virus infection. At the early stage of infection, foreign RNA was recognized by DDX1 and recruit DDX21 and DHX36 to form a complex to mediate downstream anti-viral innate immunity. At the late stage of infection, to avoid excessive immune response, host promotes DDX21 cleavage via caspase3/6. Cleaved DDX21 tends to translocate from nucleus to cytoplasm. DDX21 cleavage reduces the interaction between upstream DDX1 and downstream TRIF, and therefore suppresses the signal transduction for a late counter-regulatory effect to temper immune responses.

Supporting Information Legends

S1 Table. Primers and siRNAs used in this study

S1 Figure. DDX21 knockout inhibits IFN signaling pathway

(A) Confirmation of the genome editing by sequencing of the PCR amplicon from the DDX21 genome of the cell lines.

(B) WT and *ddx21*^{+/-} HeLa cells were seeded in 6-well plates and collected for immunoblot analysis with anti-DDX21, or anti- β -actin antibody.

(C) WT and *ddx21*^{+/-} HeLa cells were mock treated or infected with VSV at an MOI of 1, respectively. Cells were harvested at 6, 12 and 18 hpi, and detected using immunoblot analysis with anti-DDX21, anti-VSV-G, or anti- β -actin antibody.

(D) Extracellular virus yields in DDX21 knockdown and control cells.

(E-G) Virus infection experiments were performed as in C. Cells were harvested and detected using qRT-PCR with IFN- β (E), IFIT-1 (F) and MX1 (G) primers, respectively.

S2 Figure. The cleavage of DDX21 by virus infection in various cell types

(A-B) A549 cells were mock treated or infected with VSV (A) or NDV (B), respectively, at an MOI of 1. Cells were harvested at 6, 12, 18, and 24 hpi, and detected using immunoblot analysis with anti-DDX21, anti- β -actin or anti-viral-protein (VSV-G, NDV-NP) antibody.

(C-D) Huh7 (C) or THP-1 (D) cells were infected with VSV. The virus infection experiments were performed as in A.

(E) HeLa cells were infected with Sendai virus (SeV). The virus infection

experiments were performed as in A.

S3 Figure. Subcellular distribution of WT and truncated DDX21 by

nucleocytoplasmic separation assay

ddx21^{+/-} HeLa cells were transfected with either empty vector or Flag tagged WT,

D126A, 1-126 or 127-784 DDX21. At 24 h after transfection, cells were mock treated

or infected with VSV at an MOI of 1. At 18 hpi, cells were harvested for

nucleocytoplasmic separation assay, and detected using immunoblot analysis with

anti-Flag, anti- β -actin or anti-LaminB1 antibody.

A **B** **C** **D** **E** **F** **G** **H** **I** **J** **K**

A **B** **C** **D** **E** **F** **G** **H** **I** **J** **K** **H** **I** **J**

A

B

C

Caspase cleavage prediction for Q9NR30 :

>sp|Q9NR30|DDX21_HUMAN Nucleolar RNA helicase 2

```

MPGKLRSDAGLESDTAMKKGTELRKQTEEEKKEKPKSDKTEELAEETVFPKAKQVKK 60
KAEPSEVDMNSPKSKKAKKKEEPSQNDISPSTKSLRKKKEPIEKVVSSKTKKVKNEEP 120
SEEEIDAPKPKKMKKKEKEMNGETREKSPKLNKGFPHPPPCNPSEAAESENSEIEQIIP 180
VEQKEGAFSNFPISEETIKLLKGRGVTFLPFIQAKTFHHVYSGKDLIAQARTGTGTFSE 240
AIP
  
```

P1 position	PS-PP'	Sec Structure	Disorder	Prob. score	Prediction
87	PSQMD-ISPST		*****	0.715	yes
126	SEEEID-APKPK		*****	0.982	yes
160	HFSPD-CNPSE		*****	0.843	yes

D

E

F

G

H

I

A

Flag-DDX21 WT	+	+	+	+
pCMV-HA	+	-	-	-
HA-DDX21 WT	-	+	-	-
HA-DDX21 (1-125)	-	-	+	-
HA-DDX21 (127-784)	-	-	-	+

B

Flag-DDX21 (127-784)	+	+	+	+
pCMV-HA	+	-	-	-
HA-DDX21 WT	-	+	-	-
HA-DDX21 (1-125)	-	-	+	-
HA-DDX21 (127-784)	-	-	-	+

C

Flag-DDX21 (1-125)	+	+	+	+
pCMV-HA	+	-	-	-
HA-DDX21 WT	-	+	-	-
HA-DDX21 (1-125)	-	-	+	-
HA-DDX21 (127-784)	-	-	-	+

D **E**

HA-DDX1	+	+	+	+
Flag vector	+	-	-	-
Flag-DDX21 (D126A)	-	+	-	-
Flag-DDX21(127-784)	-	-	+	-
Flag-DDX21 (1-125)	-	-	-	+

F

HA-TRIF	+	+	+	+
Flag vector	+	-	-	-
Flag-DDX21 (D126A)	-	+	-	-
Flag-DDX21(127-784)	-	-	+	-
Flag-DDX21 (1-125)	-	-	-	+

G

Myc-DHX36	+	+	+	+
Flag vector	+	-	-	-
Flag-DDX21 (D126A)	-	+	-	-
Flag-DDX21(127-784)	-	-	+	-
Flag-DDX21 (1-125)	-	-	-	+