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TABLE S1: List of primers used for real time PCR

Target gene	Forward primer	Reverse primer
pIFN-β	AAATCGCTCTCTGATGTGT	TGCTCCTTTGTTGGTATCG
pIFN-γ	CCATTCAAAGGAGCATGGAT	ATCCATGCTCCTTTGAATGG
pIL-6	CACCGGTCTTGTGGAGTTTC	GTGGTGGCTTTGTCTGGATT
pIL-1β	GGGACTTGAAGAGAGAAGTGG	CTTTCCCTTGATCCCTAAGGT
pTNFα	TCACAGGGCAATGATCCC	GGGATCATTGCCCTGTGA
pMCP-1	CAGAAGAGTCACCAGCAGCA	TCCAGGTGGCTTATGGAGTC
pMX-1	TAGGCAATCAGCCATACG	GTTGATGGTCTCCTGCTTAC
pISG-15	AAATCGCTCTCCTGATGTGT	TGCTCCTTTGTTGGTATCG

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15 ## Supplementary figures

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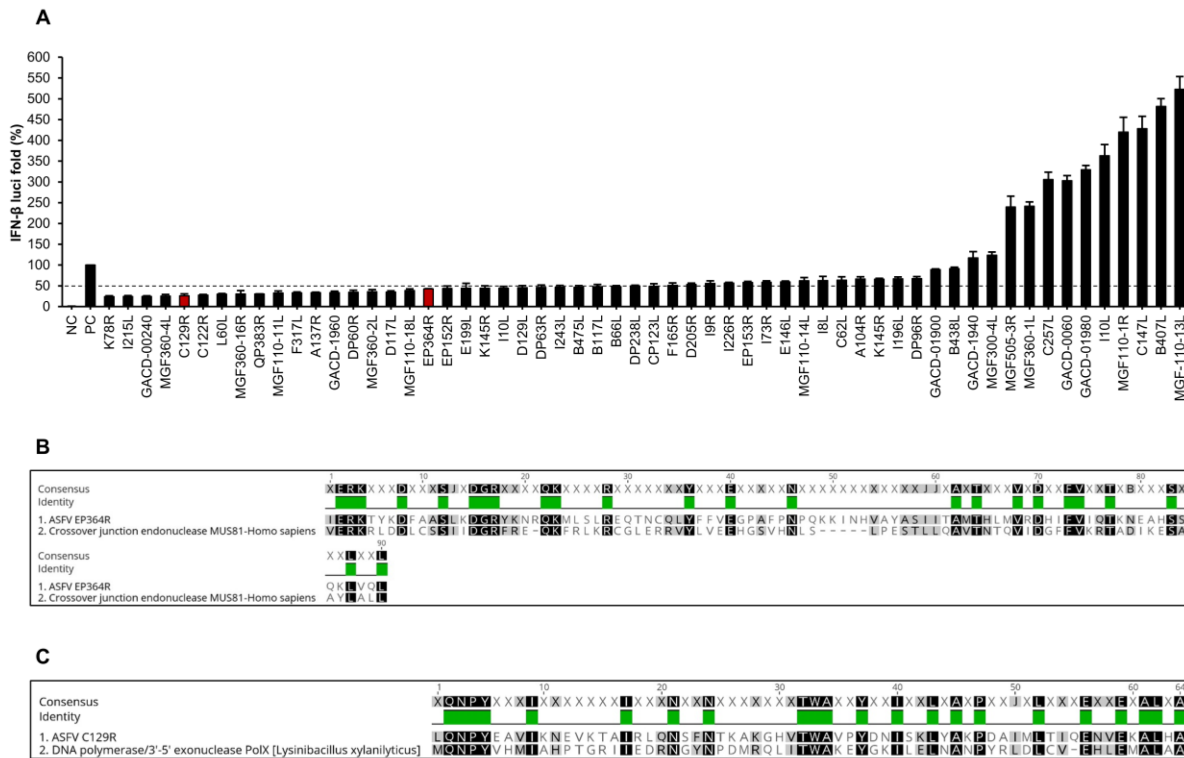

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18 **FIG S1 Screening results of ASFV genes library**

19 (A) 293-Dual™ hSTING-A162 cells were transfected with indicated individual ASFV gene for 24

20 h. Then 4 μg of 2’3’cGAMP ligand was transfected into the cells and IFN-β luciferase activity was

21 measured at 12 h post treatment. (B) Sequence alignment of ASFV EP364R with crossover

22 junction endonuclease MUS81 (*Homo sapiens*) and (C) sequence alignment of ASFV C129R with

23 DNA polymerase/ 3’-5’ exonuclease PolX (*Lysinibacillus xylanilyticus*).

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FIG S2

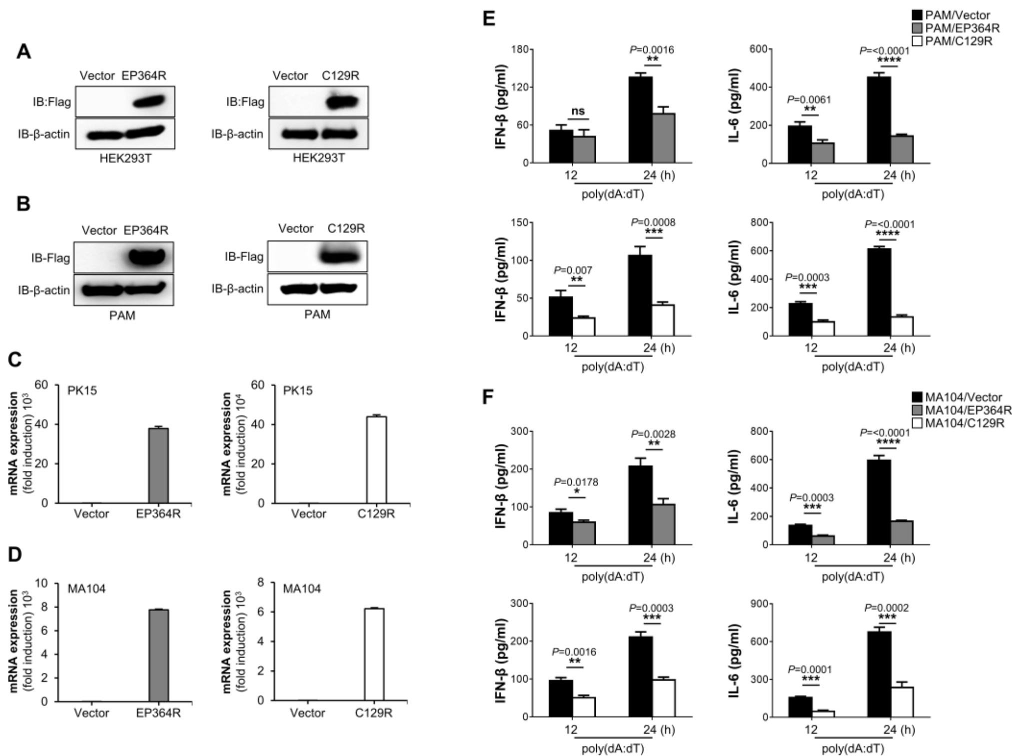

FIG S2 Overexpression of EP364R and C129R suppresses the IFN and cytokine production

Protein expression of overexpressed EP364R and C129R in HEK293T (A) and PAM cells (B). Gene transcription level of EP364R and C129R in PK-15 (C), and MA104 cells (D) Cells were transfected with an IRES vector or Flag-EP364R or Flag-C129R and cDNA was synthesized, followed by qRT-PCR. PAM cells (E) and MA104 cells (F) were transfected with Flag-EP364R and Flag-C129R plasmids and treated with poly(dA:dT) at 24 hpt. At the indicated time points, cell supernatants were collected and IFN-β and IL-6 secretions were measured. All the data are representative of at least two independent experiments, each with similar results, and the values are expressed as mean ± SD of three biological replicates. All the immunoblot data are

36 representative of at least two independent experiments, each with similar results. Student's t-test;

37 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

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FIG S3

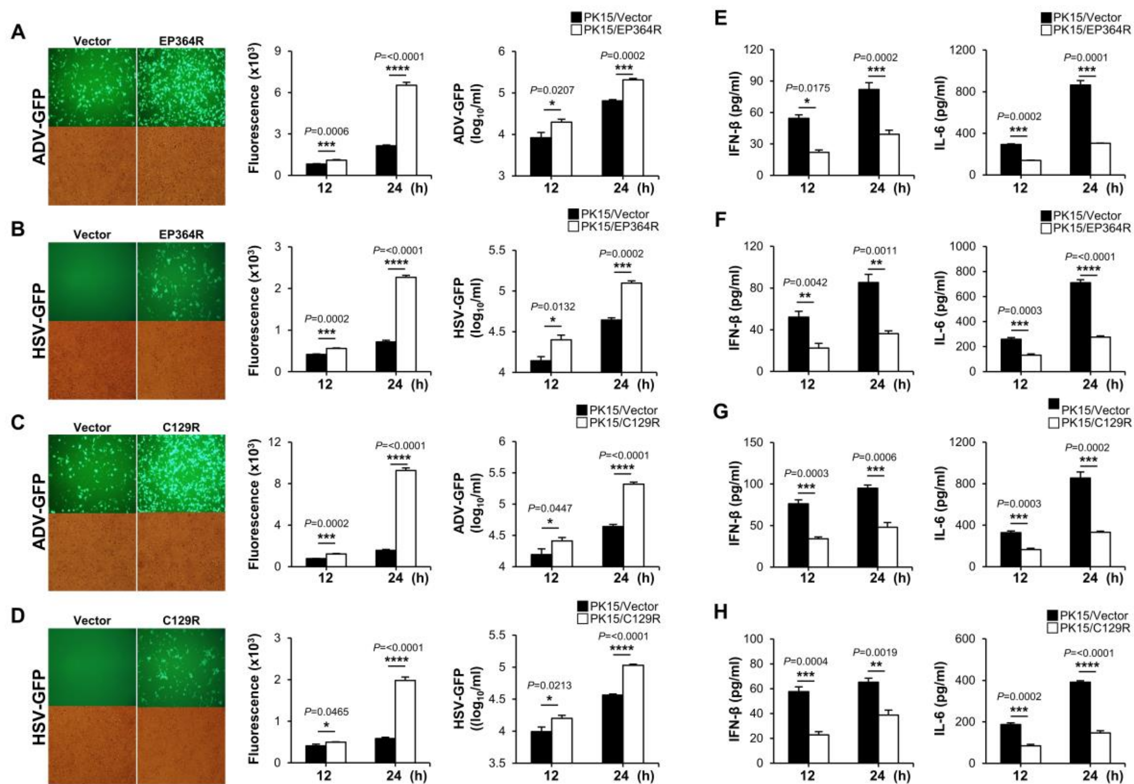


FIG S3 EP364R and C129R negatively regulate antiviral immune responses in PK-15 cells

PK-15 cells were transiently transfected with Flag-EP364R (A and B) or Flag-C129R (C and D) plasmids along with the control vector followed by the ADV-GFP (1MOI) (A and C) and HSV-GFP (1MOI) (B and D) infection at 24 hpt. Viral replication was visualized at 24 hpi by GFP expression levels using fluorescence microscopy and quantified at 12 hpi and 24 hpi by a fluorescence modulator. Virus titers of each sample were determined by plaque assay in A549 cells. Porcine IFN-β and IL-6 secretion in cell culture supernatant at 12 hpi and 24 hpi were determined by ELISA (E-H). Data are representative of at least two independent experiments, each with similar results, and the values are expressed as mean ± SD of three biological replicates. Student's t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG S4

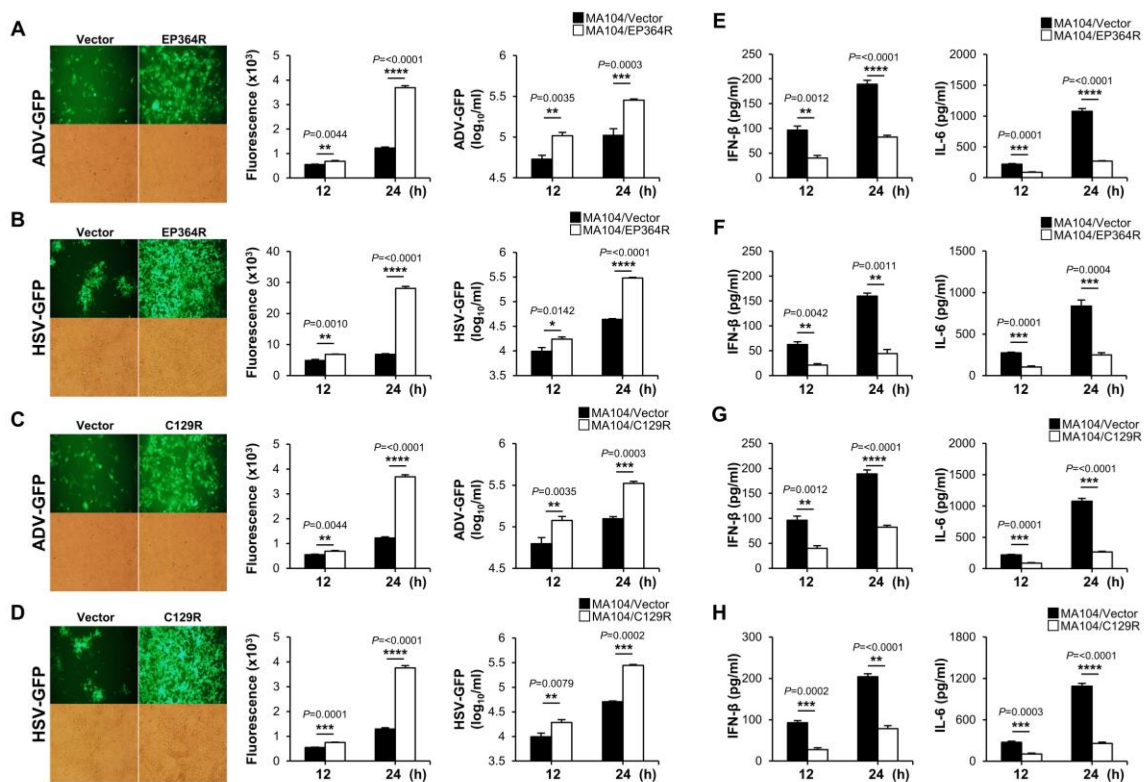


FIG S4 EP364R and C129R negatively regulate antiviral immune responses in MA104 cells

MA104 cells were transiently transfected with Flag-EP364R (A and B) or Flag-C129R (C and D) plasmids along with the control vector followed by the ADV-GFP (1MOI) (A and C) and HSV-GFP (1MOI) (B and D) infection at 24 hpt. Viral replication was determined at 24 hpi by GFP expression levels using fluorescence microscopy and quantified at 12 hpi and 24 hpi by fluorescence modulator. Virus titers of each sample were determined by plaque assay in Vero cells. Human IFN-β and IL-6 secretion in cell culture supernatant at 12 hpi and 24 hpi were determined by ELISA (E-H). Data are representative of at least two independent experiments, each with similar results, and the values are expressed as mean ± SD of three biological replicates. Student's t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG S5

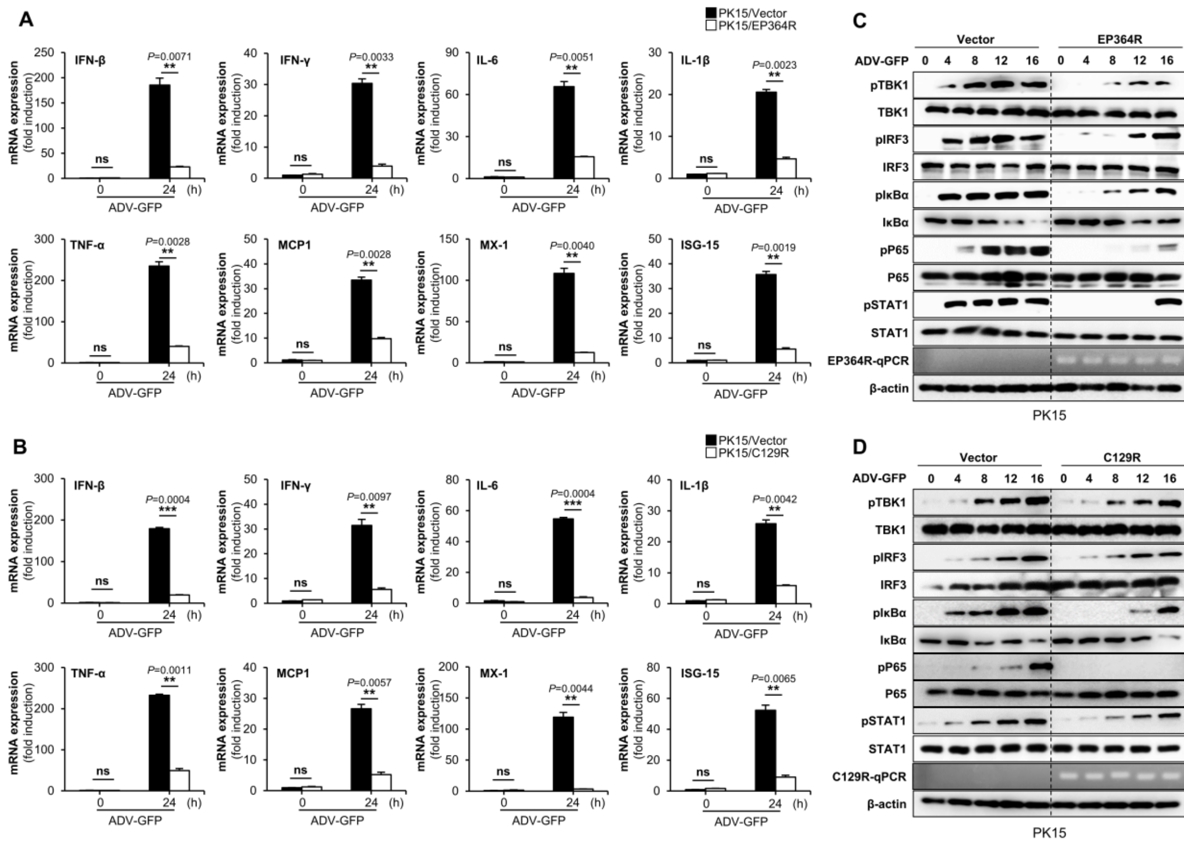
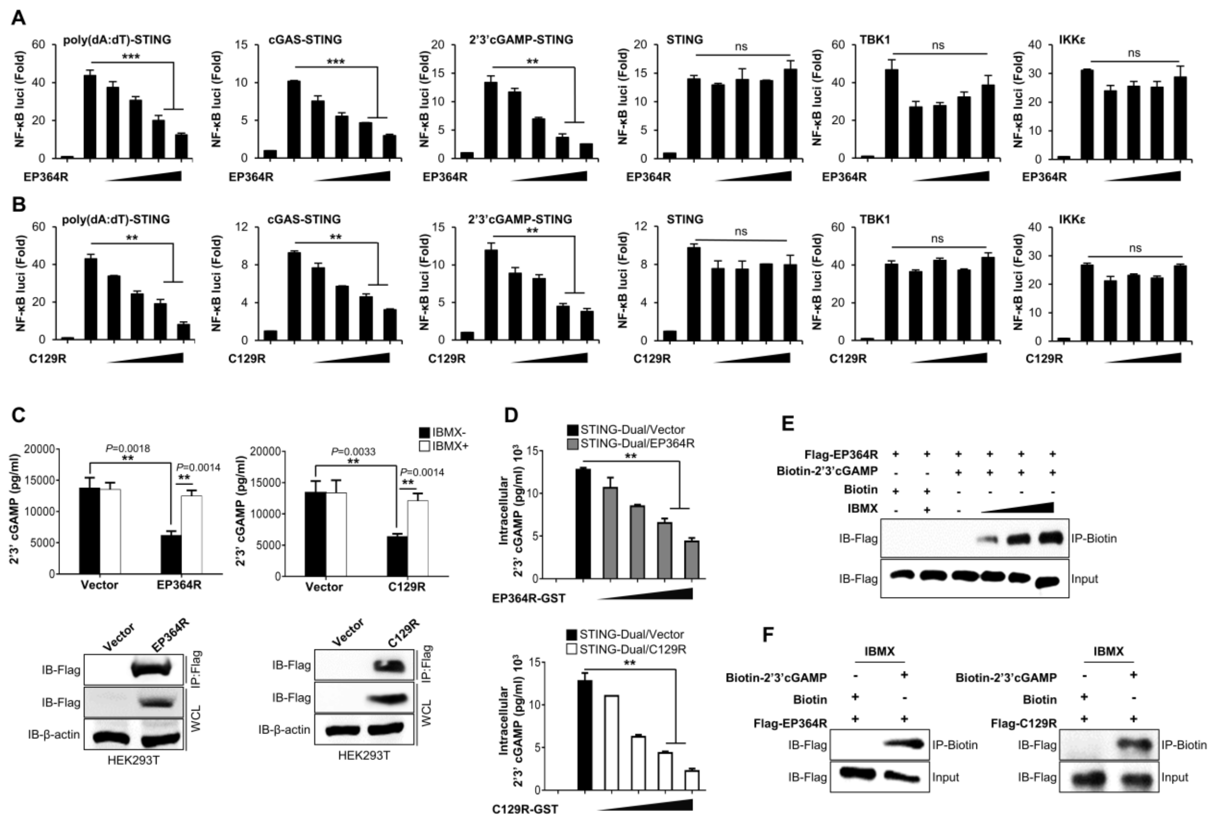


FIG S5 EP364R and C129R suppress cGAS-STING pathway and antiviral gene transcription in PK-15 cells

(A and B) PK-15 cells were transfected with Flag-EP364R or Flag-C129R plasmids and control vector plasmid. Cells were infected with ADV-GFP (1MOI) at 24 hpt, and cells were harvested at indicated time points. Then total RNA was extracted and analyzed the transcription of the indicated genes mRNA by quantitative real-time PCR. Data are representative of at least two independent experiments. (C and D) Flag-EP364R or Flag-C129R and control plasmids were transfected into PK-15 cells. Next, cells were infected with ADV-GFP (2MOI) and harvested at indicated time points. EP364R or C129R gene expression level, total and phosphorylated TBK1, IRF3, IκBα, p65, and STAT1 were measured by immunoblotting. β-actin was used as a loading control indicator.

All the data are representative of at least two independent experiments, each with similar results, and the values are expressed as mean ± SD of two biological replicates. All the immunoblot data are representative of at least two independent experiments, each with similar results. Student's t-test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

FIG S6



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97 **FIG S6 EP364R and C129R bind to 2'3'cGAMP and induce its degradation**

98 (A and B) HEK293T cells were co-transfected with an increasing dose of Flag-EP364R or Flag-
99 C129R together with firefly luciferase reporter plasmid encoding the NF-κB promoter plus TK
100 renilla plasmid and cGAS, STING, TBK1, and IKKε or stimulated with poly(dA:dT) or
101 2'3'cGAMP for 24 h and 12 h respectively. TK-Renilla was used as transfection control to
102 normalize firefly luciferase activity. STING overexpressed 293-Dual™ hSTING-A162 cells were
103 used for poly(dA:dT), cGAS, and cGAMP induced luciferase assays. Results are expressed relative
104 to those of Renilla luciferase alone (internal control). (C) *In vitro* 2'3'cGAMP degradation assay.
105 Flag-EP364R or Flag-C129R proteins plus 2.5 μM 2'3'cGAMP with 1 mM IBMX were incubated
106 for 22 h at 37^{0}C in reaction buffer followed by 2'3'cGAMP ELISA.. (D) 293-Dual™ hSTING-

107 A162 cells were co-transfected with indicated plasmids with cGAS plasmid Cells were harvested
108 at 24 hpt and intracellular 2'3'cGAMP level were measured. . (E) IBMX inhibitor dose selection
109 assay. Purified Flag-EP364R protein 2 μg was incubated with 10 μM biotin 2'3'cGAMP or 10 μM
110 biotin with reaction buffer for 2 h at 30^0C with 0.25 mM, 0.5 mM, 1 mM, and 2 mM IBMX and
111 subjected to biotin pulldown by streptavidin magnetic beads followed by immunoblotting with
112 anti-Flag antibody. (F) ASFV Flag-tagged-EP364R or C129R protein interaction with biotin-
113 conjugated 2'3'cGAMP. 2 μg of ASFV proteins were incubated with 10 μM biotin 2'3'cGAMP or
114 10 μM biotin with reaction buffer for 2 h at 30^0C with 1 mM IBMX and subjected to biotin
115 pulldown by streptavidin magnetic beads followed by immunoblotting with anti-Flag antibody.
116 All the luciferase data are representative of at least two independent experiments, each with similar
117 results, and the values are expressed as mean ± SD of two biological replicates. All the ELISA
118 data are representative of at least two independent experiments, each with similar results, and the
119 values are expressed as mean ± SD of three biological replicates. All the immunoblot data are
120 representative of at least two independent experiments, each with similar results. Student's t-test;
121 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
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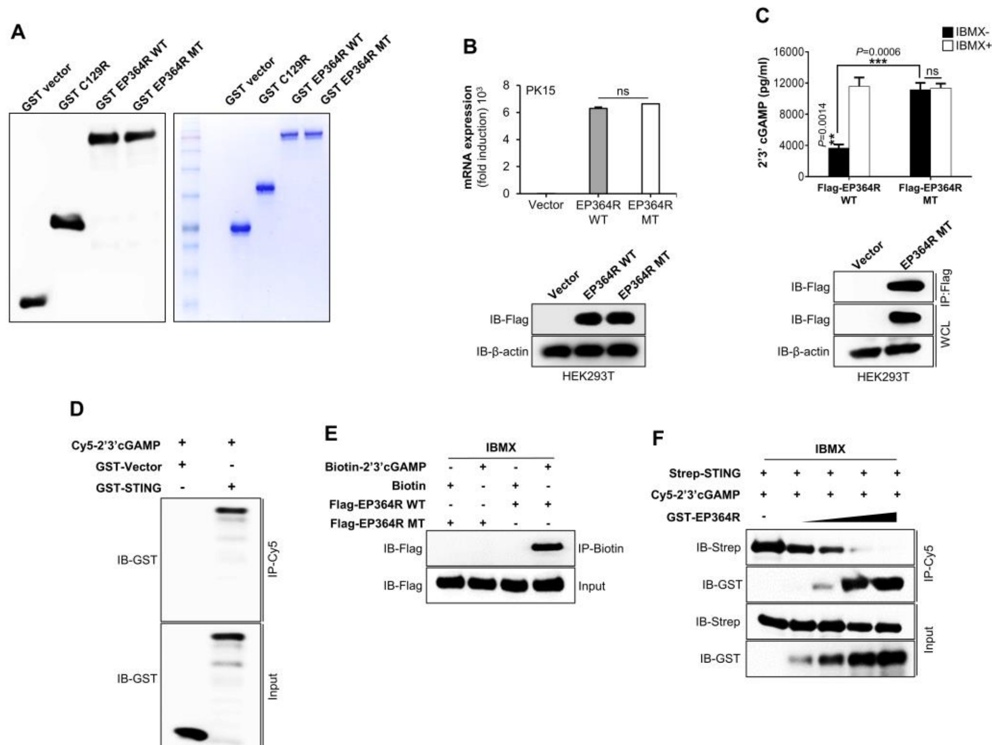
FIG S7



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131 **FIG S7 GST protein purification and STING-cGAMP interaction competition**

132 (A) Purified GST protein was confirmed by western blot analysis and comassie staining of SDS-
133 PAGE. (B) Overexpression of Flag-EP364R-WT and Flag-EP364R-MT in PK-15 cells and
134 HEK293T cells. PK-15 cells were stably transfected with an Flag vector or Flag-EP364R-WT and
135 Flag-EP364R-MT and cDNA synthesized for qRT-PCR. Overexpression of Flag-EP364R-WT and
136 its mutant plasmid was confirmed by HEK293T cell stably transfected with an IRES-Flag vector
137 or Flag-EP364R-WT Flag-EP364R-MT immunoblotting with anti-Flag antibodies and β-Actin
138 was used to confirm equal loading of proteins. (C) *In vitro* 2'3'cGAMP degradation assay. Flag-
139 EP364R-WT or its mutant EP364R-MT proteins plus 2.5 μM 2'3'cGAMP with 1 mM IBMX were
140 incubated 22 h at 37^{0}C in reaction buffer followed by 2'3'cGAMP ELISA to quantify the remaining

141 2'3'cGAMP level in incubated samples. (D) *In vitro* STING – 2'3'cGAMP binding assay.
142 Immunoprecipitated 2 μg of Strep-STING protein were incubated with 10 μM of Cy5 conjugated
143 2'3'cGAMP with reaction buffer for 2 h at 30^{0}C with 1 mM IBMX and subjected toCy5 pulldown
144 followed by immunoblotting with antiGST antibody. (E) *In vitro* 2'3'cGAMP binding assay. Flag
145 immunoprecipitated 2 μg of ASFV EP364R-WT or EP364R-MT proteins were incubated in 10
146 μM 2'3'cGAMP-biotin or 10 μM biotin with reaction buffer for 2 h at 30^{0}C with 1 mM IBMX
147 followed by streptavidin pulldown (F) *In vitro* 2’3’cGAMP binding competition assay. The
148 increasing amount of GST purified protein of EP364R-WT (0.25 μg, 0.5 μg, 1 μg, 2 μg) and GST
149 vector protein as control (1.75 μg, 1.5 μg, and 1 μg) with a constant amount of Strep-STING
150 protein (1 μg) incubated with 10 μM Cy5 conjugated 2'3'cGAMP in reaction buffer for 2 h at 30^{0}C
151 with1mM IBMX. Incubated protein mixture immunoprecipitated by anti Cy5 antibody followed
152 by immunoblotting with anti-GST and anti-Strep antibodies. All the ELISA data are representative
153 of at least two independent experiments, each with similar results, and the values are expressed as
154 mean ± SD of three biological replicates. All the immunoblot data are representative of at least
155 two independent experiments, each with similar results. Student’s t-test; * $p < 0.05$; ** $p < 0.01$;
156 *** $p < 0.001$; ns, not significant.
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