Supplementary Materials for

CD-NTase family member MB21D2 promotes cGAS-mediated antiviral and antitumor immunity

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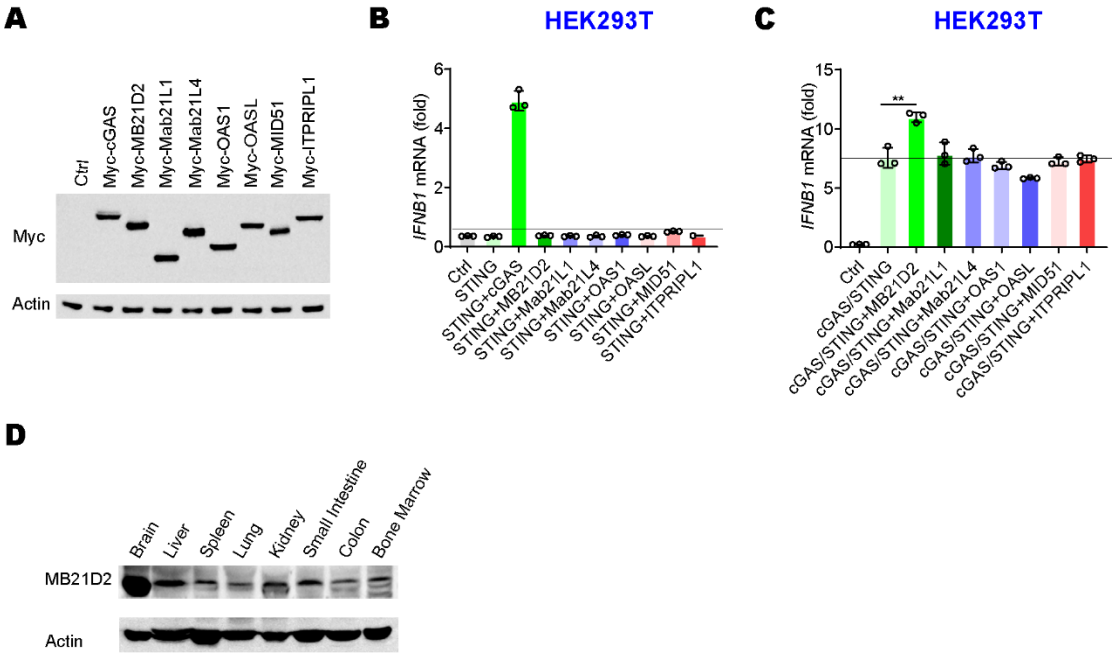
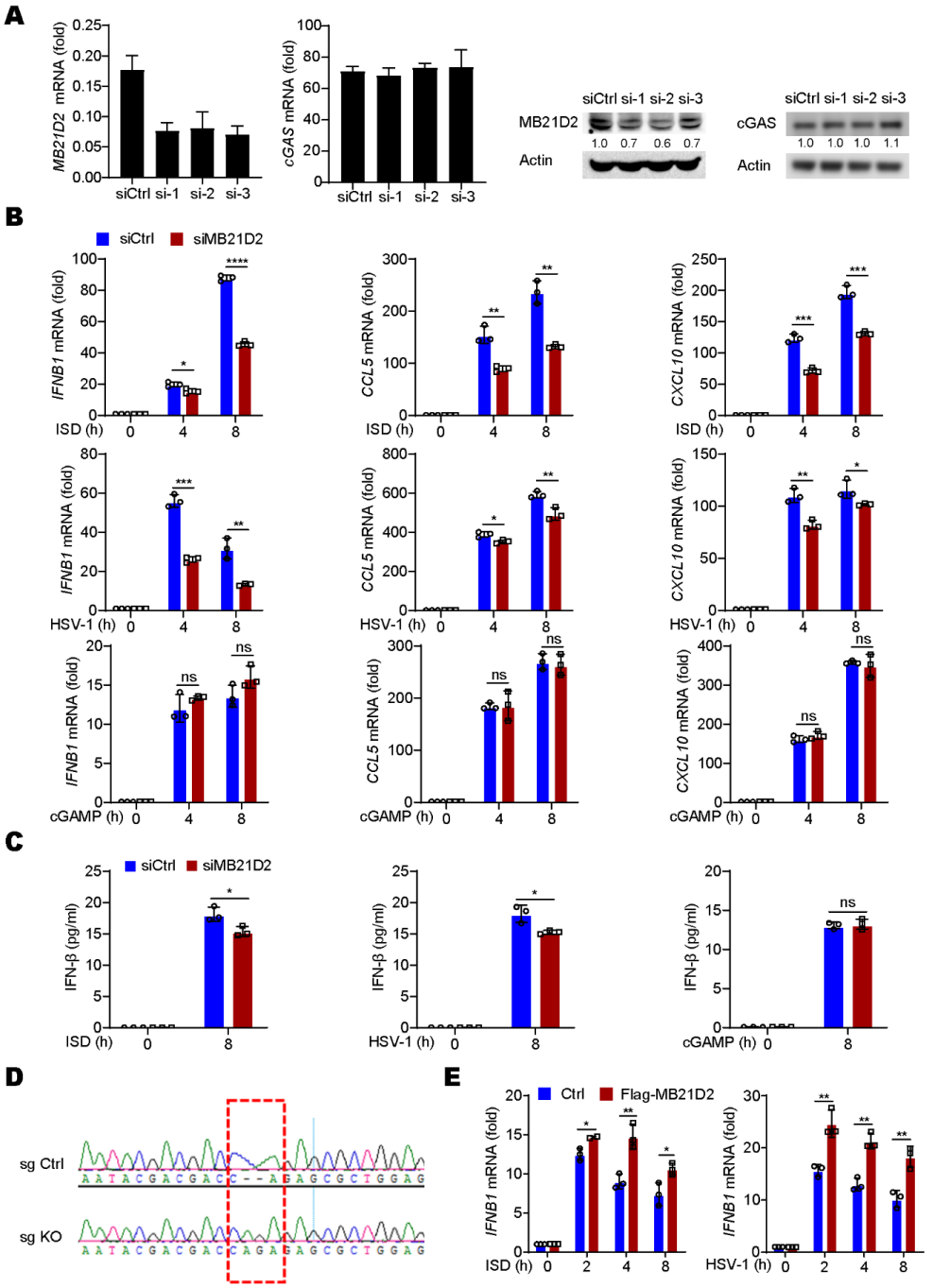


Supplementary Fig. S1 MB21D2 facilitates cGAS-mediated IFN-β production.

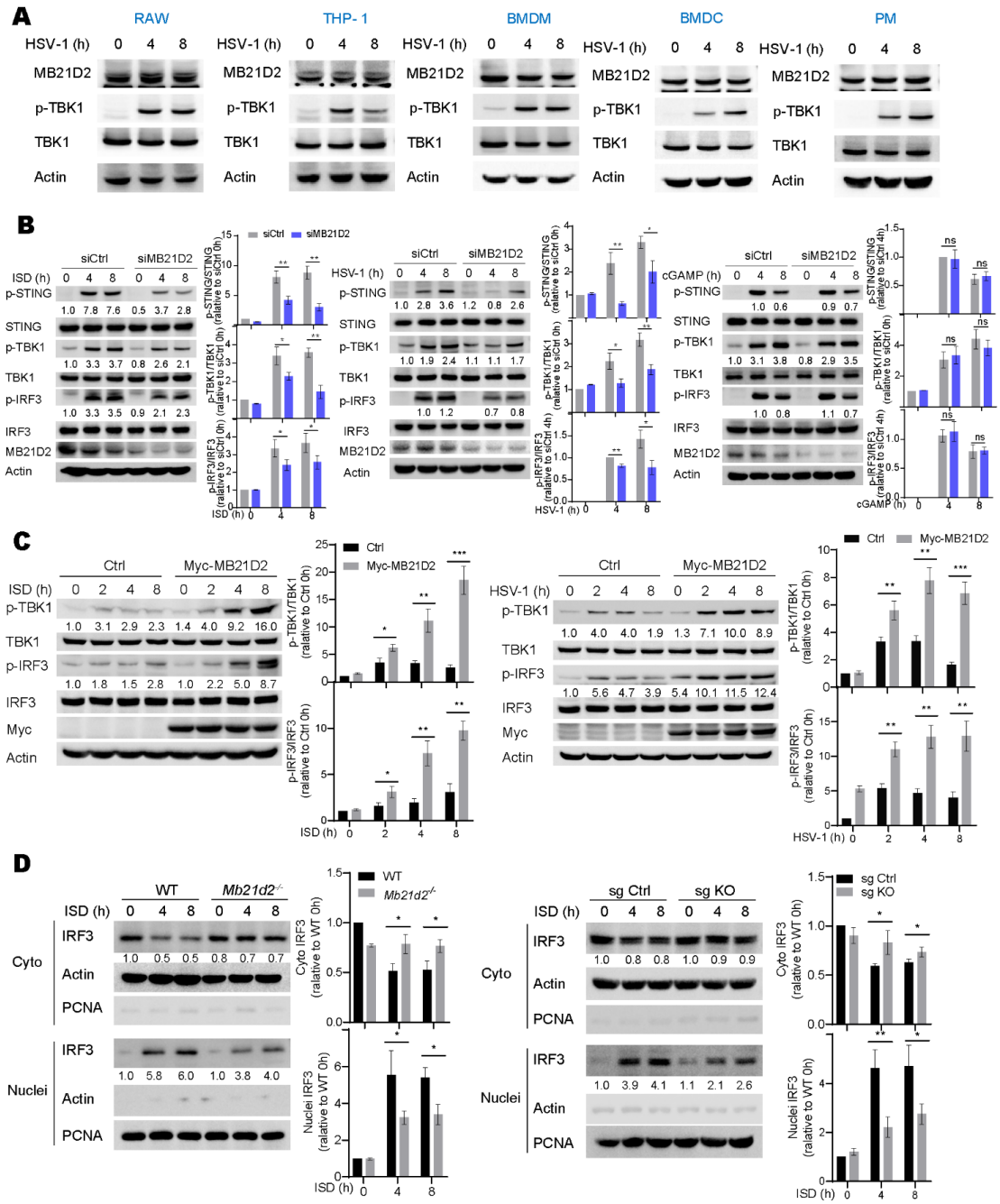
A Western blot analysis of each CD-NTase family member expression in HEK293T cells. **B** HEK293T cells were transfected with different CD-NTase family member expression plasmid together with STING expression plasmid for 24 h. Expression of *IFNB1* mRNA was measured by RT-PCR, n=3. **C** HEK293T cells were transfected with different CD-NTase family member expression plasmid together with cGAS and STING expression plasmid for 24 h. Expression of *IFNB1* mRNA was measured by RT-PCR, n=3. **D** Western blot analysis of MB21D2 in different mice organs. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's t test (**C**) (**p<0.01).



Supplementary Fig. S2 Knockdown of MB21D2 attenuates the production of IFN-β in THP-1 cells.

A The knockdown efficiency of MB21D2 and cGAS in THP-1 cells was confirmed by RT-PCR (left) and Western blot (right) after transfection with MB21D2 siRNAs. **B** RT-PCR analysis of *IFNB1*, *CCL5* and *CXCL10* in THP-1 cells transfected with control siRNA or MB21D2 siRNA for 48 h, followed by stimulated with ISD or cGAMP, or infected with HSV-1 for indicated times, *n*=3. **C** ELISA quantification of IFN-β secretion level in THP-1 cells treated as in (**B**), *n*=3. **D** The DNA sequencing

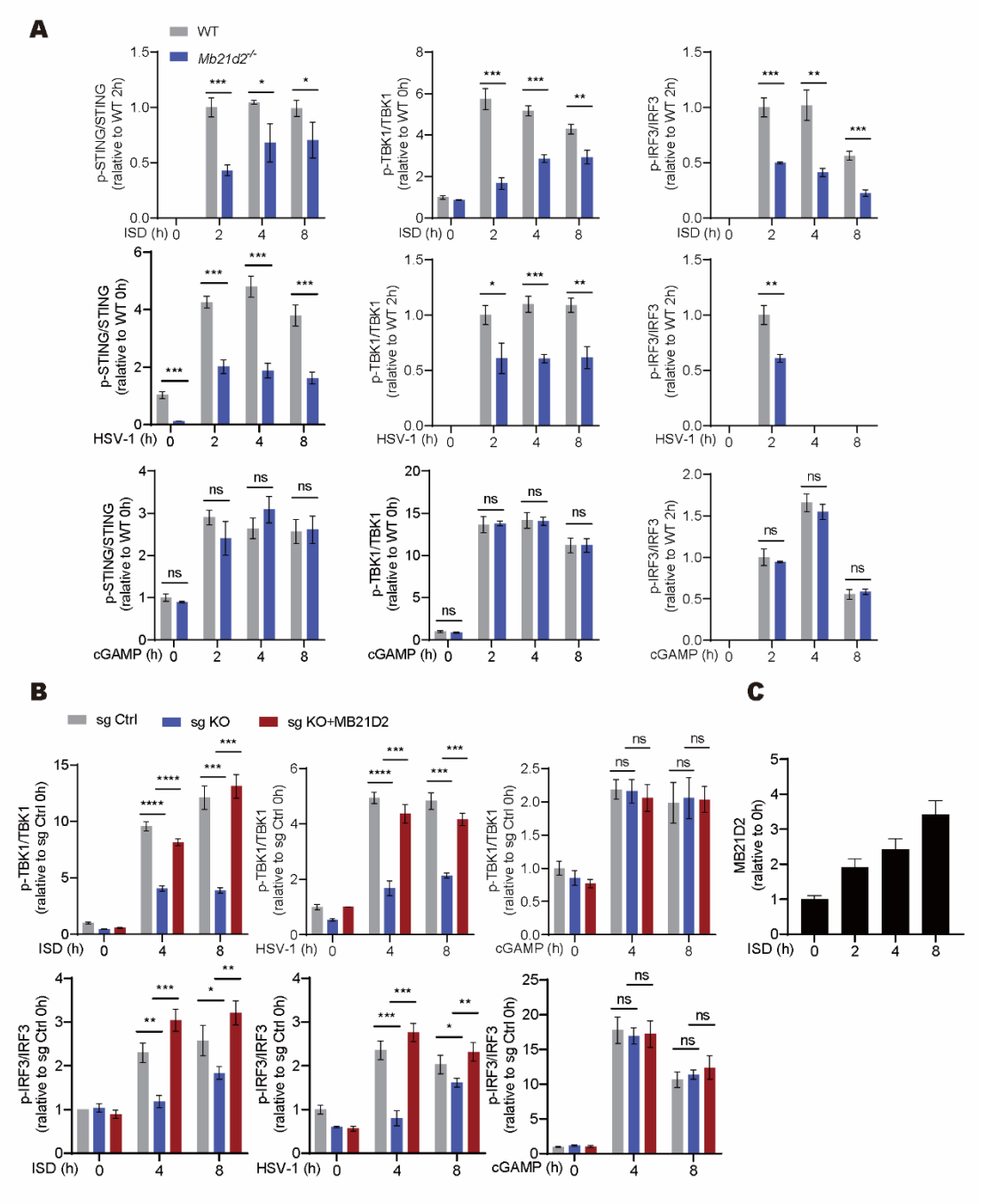
results of MB21D2 expression in the sg Ctrl and sg KO HeLa cells. **E** HeLa cells were overexpressed with Ctrl or Flag-MB21D2 plasmid for 24 h, followed by stimulated with ISD, or infected with HSV-1 for indicated times. Expression of *IFNB1* mRNA was measured by RT-PCR, n=3. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's t test (**B**, **C**, and **E**) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



Supplementary Fig. S3 MB21D2 promotes cGAS-mediated IFN-β signaling.

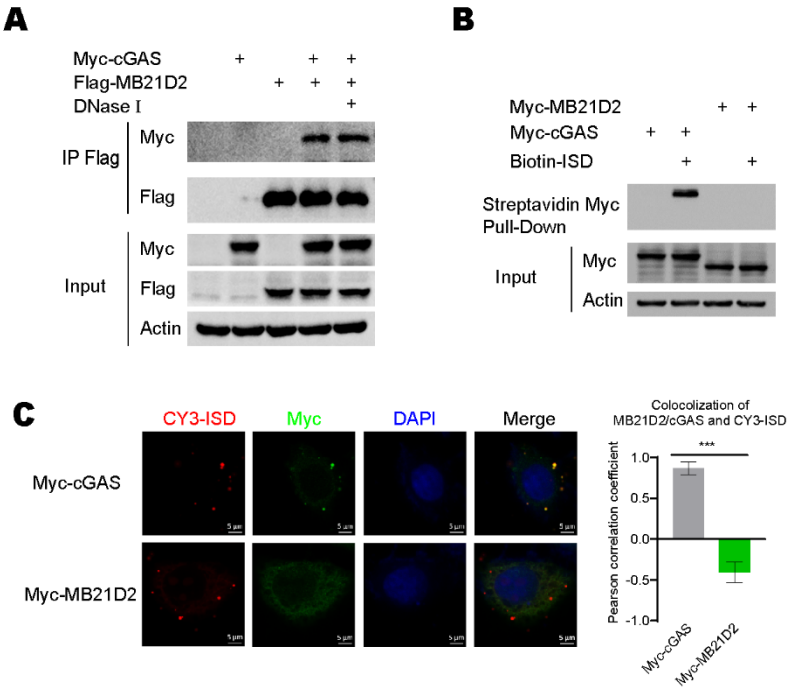
A Western blot analysis of MB21D2 in different myeloid cells upon HSV-1 infection. **B** Western blot analysis of p-STING, p-TBK1 and p-IRF3 in THP-1 cells transfected with control siRNA or MB21D2 siRNA for 48 h, followed by stimulated with ISD or cGAMP, or infected with HSV-1 for indicated times. Densitometric quantification was measured by Image J. **C** Western blotting of p-TBK1 and p-IRF3 in HeLa cells overexpressed with Myc-MB21D2 plasmid or not, followed by ISD stimulation or HSV-1 infection for indicated times. Densitometric quantification was measured by

Image J. **D** Western blot analysis of IRF3 protein in nuclear (nuclei) and cytoplasmic (cyto) fractions in mice macrophages (left) and HeLa cells (right) upon ISD stimulation. Actin served as cytoplasmic control. PCNA served as nuclear protein control. Densitometric quantification was measured by Image J. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's *t* test (**B-D**) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).



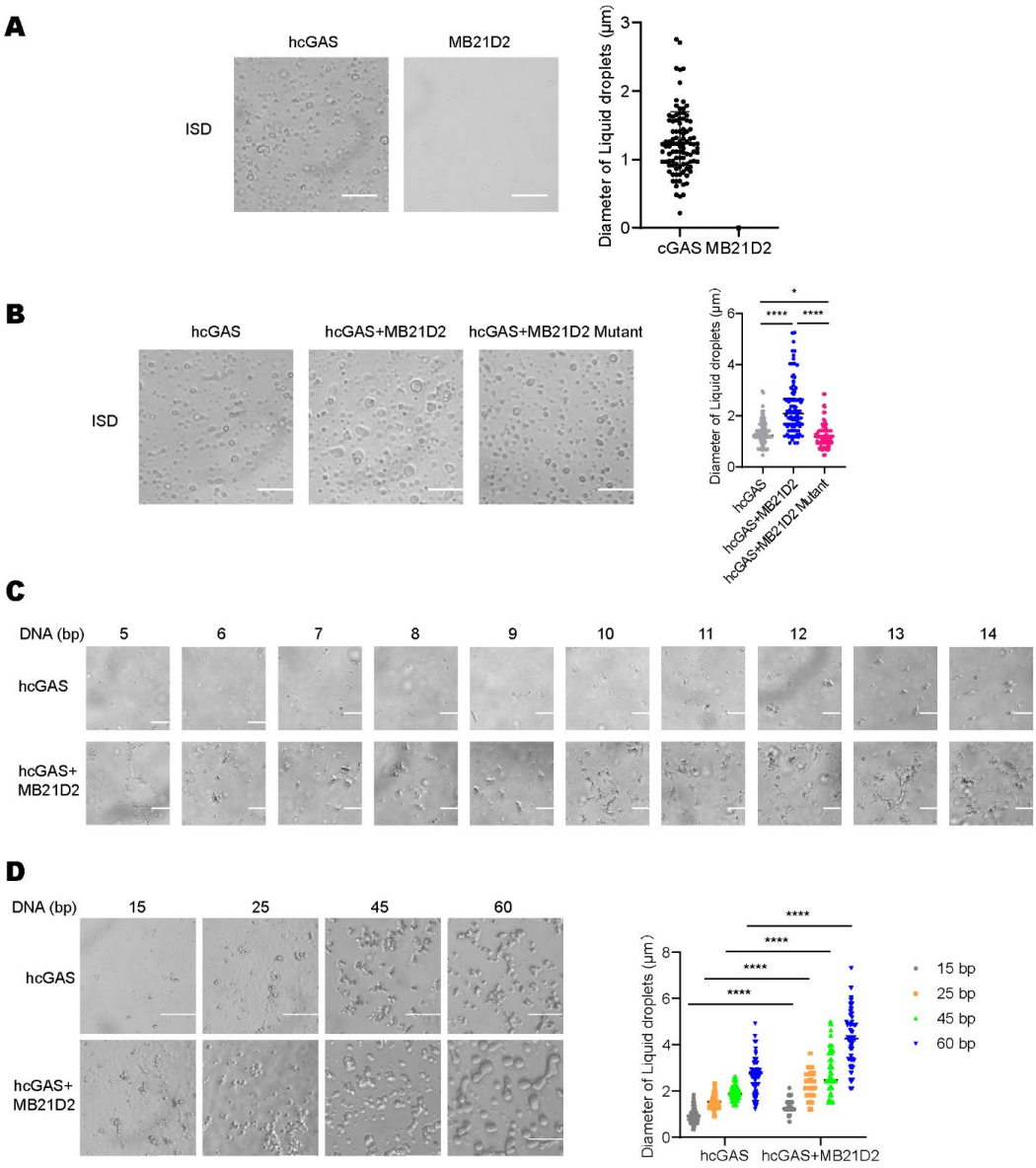
Supplementary Fig. S4 MB21D2 positively regulates cGAS-mediated IFN-β signaling.

A Densitometric quantification of p-STING, p-TBK1 and p-IRF3 as shown in (**Fig. 2A**) was measured by Image J. **B** Densitometric quantification of p-TBK1 and p-IRF3 as shown in (**Fig. 2B**) was measured by Image J. **C** Densitometric quantification of MB21D2 as shown in (**Fig. 3B**) was measured by Image J. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's *t* test (**A-C**) (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).



Supplementary Fig. S5 MB21D2 could not bind to DNA.

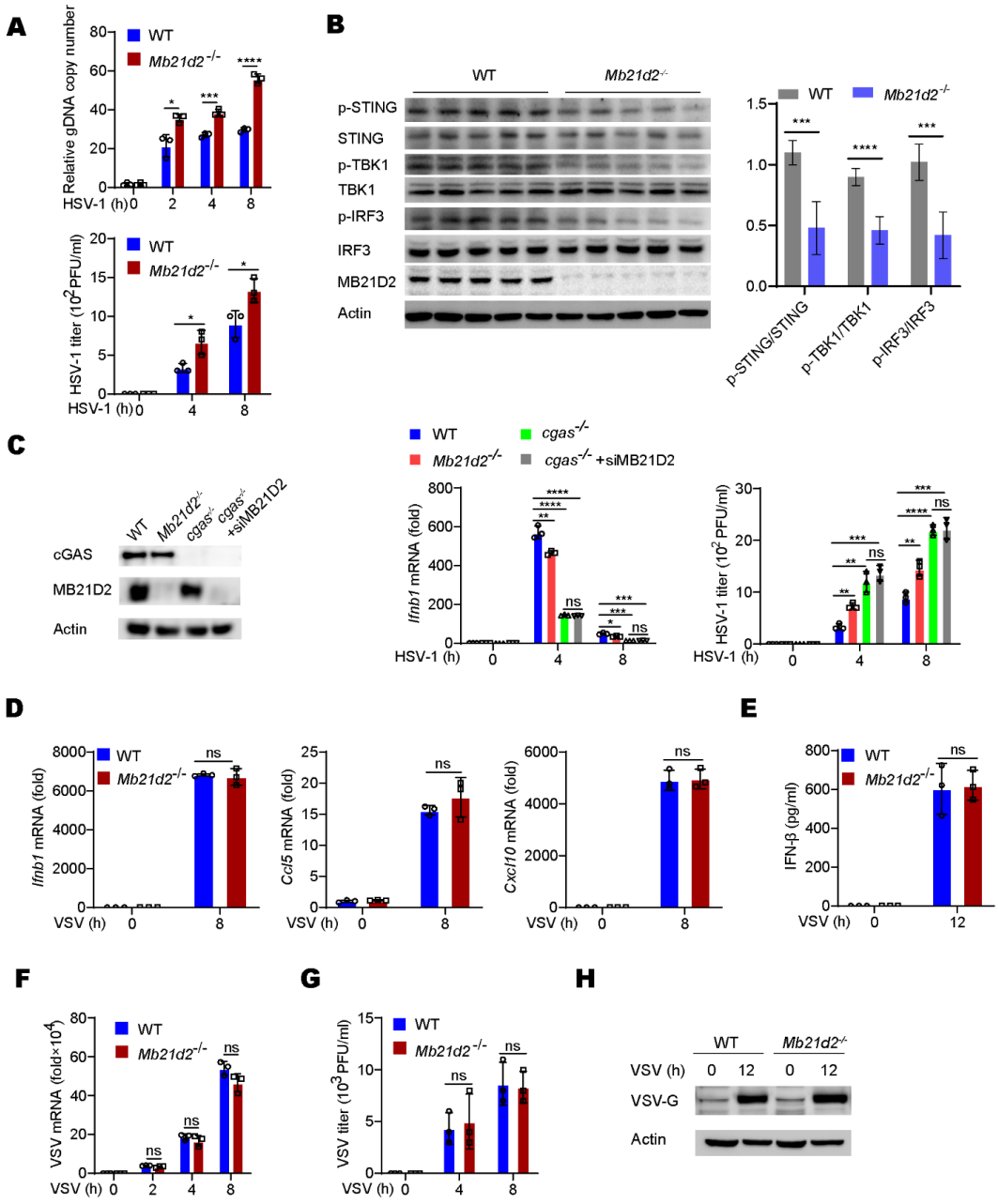
A HEK293T cells were overexpressed with Myc-cGAS or Myc-MB21D2 for 24 h, followed by DNase I (1 mg/mL) treatment for 2 h. Coimmunoprecipitations and immunoblots were performed with the indicated antibodies. **B** HEK293T cells were overexpressed with Myc-cGAS or Myc-MB21D2 for 24 h, followed by Biotin-ISD stimulation for 4 h. The DNA-binding capacity of cGAS and MB21D2 was analyzed by Streptavidin Pull-Down assay. **C** HeLa cells were overexpressed with Myc-cGAS or Myc-MB21D2 for 24 h, followed by CY3-ISD stimulation for 4 h. The DNA-binding capacity of cGAS and MB21D2 was analyzed by microscopy images. Pearson correlation coefficient was analyzed by Image J. Scale bar, 5 μm. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's *t* test (**C**) (***p<0.001).



Supplementary Fig. S6 MB21D2 promotes the liquid phase condensation of cGAS.

A Bright-field images of phase separation of 10 μM hcGAS FL or 10 μM MB21D2 with 10 μM ISD. Scale bar, 15 μm. Diameter of Liquid droplets was measured by Image J. **B** Bright-field images of phase separation of 10 μM hcGAS FL in the presence of 10 μM MB21D2 FL or MB21D2 Mutant. Scale bar, 15 μm. Diameter of Liquid droplets was measured by Image J. **C** Representative bright-field images of phase separation experiments shown in (**Fig. 6E**). Scale bar, 15 μm. **D** Bright-field images of phase separation of 10 μM hcGAS FL and 10 μM indicated length DNA in the presence or absence of 10 μM MB21D2. Diameter of Liquid droplets was

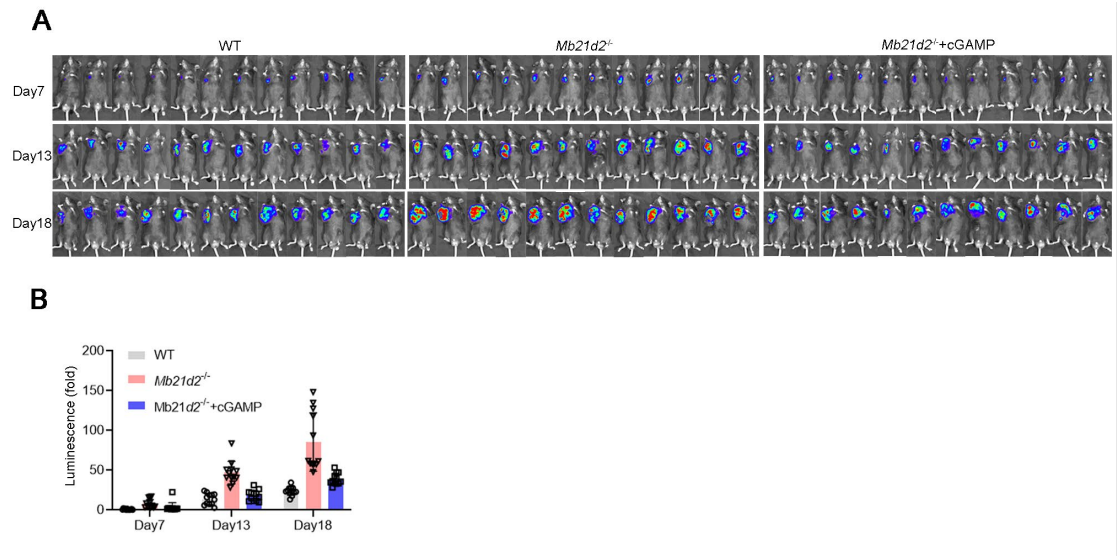
measured by Image J. Scale bar, 20 μm. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's *t* test (**B, D**) (*p<0.05, ****p<0.0001).



Supplementary Fig. S7 MB21D2 do not regulate RNA virus-induced immune response.

A The gDNA copy number (top) and viral titers (bottom) of HSV-1 in WT or *Mb21d2*$^{-/-}$ mice peritoneal macrophages after infected with HSV-1 for indicated times, *n*=3. **B** Western blot analysis of p-STING, p-TBK1 and p-IRF3 in the brains of WT or *Mb21d2*$^{-/-}$ mice 3 d after intravenous injection of HSV-1 (1×10^7 PFU per mouse). Densitometric quantification was measured by Image J. **C** The protein level of MB21D2 or cGAS (left), mRNA level of *Ifnb1* (middle) and HSV-1 titers (right) in

WT, *Mb21d2*$^{-/-}$, *cgas*$^{-/-}$, *cgas*$^{-/-}$+siMB21D2 peritoneal macrophages after infected with HSV-1 for indicated times, *n*=3. **D** RT-PCR analysis of *Ifnb1, Ccl5,* and *Cxcl10* mRNA in WT or *Mb21d2*$^{-/-}$ mice peritoneal macrophages after VSV infection for indicated times, *n*=3. **E** ELISA quantification of IFN-β secretion level in WT or *Mb21d2*$^{-/-}$ mice peritoneal macrophages after VSV infection for 12 h, *n*=3. **F, G** The expression of *VSV* mRNA (**F**), and VSV titers (**G**) in WT or *Mb21d2*$^{-/-}$ mice peritoneal macrophages after VSV infection for indicated times, *n*=3. **H** Western blot analysis of VSV-G protein in WT or *Mb21d2*$^{-/-}$ mice peritoneal macrophages after VSV infection for 12 h. Data are shown as mean ± SD and were analyzed by unpaired two tailed Student's *t* test (**A**-**G**). (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).



Supplementary Fig. S8 MB21D2 positively regulates antitumor immunity.

A Representative bioluminescent images of tumors are shown for WT, *Mb21d2*$^{-/-}$, *Mb21d2*$^{-/-}$administration with cGAMP (10 mg/kg) mice implanted with B16-Luc cells, *n*=12. **B** Bioluminescent quantification of tumors in WT, *Mb21d2*$^{-/-}$, *Mb21d2*$^{-/-}$ administration with cGAMP (10 mg/kg) mice implanted with B16-Luc cells for indicated times, *n*=12.

Supplementary Table S1 Primers used in this study

Gene	Species	Forward primer (5'-3')	Reverse primer (5'-3')
Ifnb1	Mouse	AGTTACACTGCCTTTGCC	GTTGAGGACATCTCCCAC
IFNB1	Human	CAACAAGTGTCTCCTCCAAAT	TCTCCTCAGGGATGTCAAAG
Ccl5	Mouse	CCTCACCATATGGCTCGGAC	TCTTCTCTGGGTTGGCACAC
CCL5	Human	CCCCATATTCCTCGGACACC	GCTGTCCCTCTCTCTTTGGC
Cxcl10	Mouse	CCAAGTGCTGCCGTCATTTT	GATAGGCTCGCAGGGATGAT
CXCL10	Human	TGCCATTCTGATTTGCTGCC	TGATGGCCTTCGATTCTGGA
Actin	Mouse	CCACACCCGCCACCAGTTCG	TACAGCCCGGGGAGCATCGT
ACTIN	Human	GGAAATCGTGCGTGACATTAA	AGGAAGGAAGGCTGGAAGAG
VSV-G		CAAGTCAAAATGCCCAAGAGTCACA	TTTCCTTGCATTGTTCTACAGATGG
HSV-1		TGGGACACATGCCTTCTTGG	ACCCTTAGTCAGACTCTGTTACTTACCC

Supplementary Table S2 Oligonucleotides used in this study

oligonucleotide	Forward primer (5'-3')	Reverse primer (5'-3')
MB21D2 siRNA1	GGACUUAGAUGAGCUUAAUTT	AUUAAGCUCAUCUAAGUCCTT
MB21D2 siRNA2	GUCGCAUGUUGUAUGAUAUTT	AUAUCAUACAACAUGCGACTT
MB21D2 siRNA3	GCUCCUACAAGGGUAAGAATT	UUCUUACCCUUGUAGGAGCTT
ds DNA 5 bp	ACATC	GATGT
ds DNA 6 bp	ACATCT	AGATGT
ds DNA 7 bp	ACATCTA	TAGATGT
ds DNA 8 bp	ACATCTAG	CTAGATGT
ds DNA 9 bp	ACATCTAGT	ACTAGATGT
ds DNA 10 bp	ACATCTAGTA	TACTAGATGT
ds DNA 11 bp	ACATCTAGTAC	GTACTAGATGT
ds DNA 12 bp	ACATCTAGTACA	TGTACTAGATGT

ds DNA 13 bp	ACATCTAGTACAT	ATGTACTAGATGT
ds DNA 14 bp	ACATCTAGTACATG	CATGTACTAGATGT
ds DNA 15 bp	AAACAACACAACAAA	TTTGTTGTGTTGTTT
ds DNA 25 bp	AAAACAAACAACACAACAAACAAAA	TTTTGTTTGTTGTGTTGTTTGTTTT
ds DNA 45 bp (ISD)	TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA	TGTAGATCATGTACAGATCAGTCATAGATCACTAGTAGATCTGTA
ds DNA 55 bp	TCGATACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACAATCACT	AGTGATTGTAGATCATGTACAGATCAGTCATAGATCACTAGTAGATCTGTATCGA
ds DNA 60 bp	TAAGACACGATGCGATAAAATCTGTTTGTAAAATTTATTAAGGGTACAAATTGCCCTAGC	GCTAGGGCAATTTGTACCCTTAATAAATTTTACAAACAGATTTTATCGCATCGTGTCTTA