Supplementary Materials for

Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type-I interferon pathway

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Materials and Methods

General methods: General methods were described Wu et al (*1*) except for the following information. cDNA clones for m-cGAS (E330019A16; accession #BC145653) and h-cGAS (C6orf150; accession #BC108714) were purchased from Open Biosystems. The coding sequences for cGAS, as well as those for DAI, IFI16, DDX41 and MAVS, were subcloned into pcDNA3 in-frame with an N-terminal Flag tag. The lentiviral shRNA vector for knocking down mouse STING and the generation of L929/shSTING stable cells were described previously(*2*). The same vector and method were employed to establish L929/sh-cGAS and THP1/sh-cGAS cell lines using the following targeting sequences (only sense strand sequence is shown): m-cGAS-a: 5'-GGATTGAGCTACAAGAATA, and m-cGAS-b: 5'- GCTGTAACACTTCTTATCA-3'; h-cGAS-a: 5'-GGAAGGAAATGGTTTCCAA and h-cGAS-b: 5'-GCCTTCTTTCACGTATGTA-3'. Primers for quantitative RT-PCR (q-RT-PCR) were: m-cGAS, sense: ACCGGACAAGCTAAAGAAGGTGCT, anti-sense: GCAGCAGGCGTTCCACAACTTTAT; h-cGAS, sense: GGGAGCCCTGCTGTAACACTTCTTAT, anti-sense: CCTTTGCATGCTTGGGTACAAGGT. siRNA oligos were purchased from Sigma and the sense strand sequences targeting the following mouse genes were: DDX41: UGACAUGCCUGAAGAGAUATT; p204: AGAAAACAGUGAACCGAAATT; STING: CGAAAUAACUGCCGCCUCATT; m-cGAS-a: GGAUUGAGCUACAAGAAUATT; m-cGAS-b: GCUGUAACACUUCUUAUCATT. The antibody against human cGAS (C6orf150) was purchased from Sigma.

In vitro assay for cGAS activity

To measure cGAS activity, cell extracts or fractions from chromatography were mixed with buffer A (20 mM Hepes, pH7.2, 5 mM $MgCl_2$, 2 mM ATP, 2 mM GTP, 0.1 mM EGTA), in the presence or absence of 0.1 mg/ml herring testis DNA (HT-DNA). After incubation at 37°C for 45 min, the mixture was heated at 95°C for 5 min, centrifuged at 10,000 x g for 3 min, then the heat-resistant supernatant was mixed with PFO permeabilized THP1 or Raw264.7 cells to measure IRF3 activation.

Purification of cGAS from Cell Extracts

Cytosolic extracts (S100) of L929/shSTING were used to purify endogenous cGAS through three different routes (see fig. S1A), each route using approximately 250 mg of starting materials [from ~200 plates (15 cm ID) of cultured cells]. In route I, S100 was loaded onto a Heparin column equilibrated with buffer B [20 mM Tris-HCl, pH7.4, 0.02% CHAPS, 0.5 mM DTT, and 0.1 mM PMSF], and eluted sequentially with 0.5 M and 1.0 M NaCl in buffer B. The 1.0 M eluate, which contained the cGAS activity, was further fractionated with a 0.5 M-1.0 M gradient on a heparin column. Active fractions were pooled, concentrated and subjected to gel filtration on a Superose 6 PC 3.2/30 column in buffer C [20mM Tris-Cl, pH8.0, 150mM NaCl, 0.02% CHAPS, 0.5mM DTT, 0.1mM PMSF] using the ETTAN micropurification system (GE Healthcare). Active fractions were loaded onto a Mono Q PC 1.6/5 column (GE Healthcare) equilibrated with buffer B and eluted with a 0-0.5 M NaCl gradient. In route II, the 1.0 M eluate from the Heparin column was loaded onto a hydroxyapatite column (Bio-Rad) equilibrated with a buffer containing 10 mM KPO_4, pH7.0 (a mixture of K_2HPO_4 and KH_2PO_4), and eluted with 0.125 M KPO_4, pH7.0. The eluate was further fractionated with the Mono Q PC column as described in route I. Active fractions were pooled and loaded onto a Mono S PC 1.6/5 column in buffer D [20 mM Tris-Cl, pH 8.3, 0.02% CHAPS, 0.5 mM DTT, 0.1 mM PMSF], and eluted with a 0-1.0 M NaCl gradient in buffer D. In route III, active fractions from mono Q PC of route II were applied to biotin-ISD (Sigma) immobilized on monomeric Avidin UltraLink beads (Pierce), which was equilibrated with buffer E [20 mM Tris-Cl, pH7.4, 100 mM NaCl, 0.02% CHAPS]. After extensive washing with the same buffer, bound proteins were eluted with 2 mM biotin in buffer E.

Expression and Purification of Recombinant Proteins

cDNA encoding full-length human or mouse cGAS was inserted into a modified pET28a vector containing an in-frame His_6-SUMO tag. The *E. coli* strain BL21/pLys harboring the plasmid was induced with 0.5 mM IPTG at 18°C overnight. *E coli* was resuspended and sonicated in lysis buffer I [50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.2 mM PMSF] and centrifuged at 100,000 x g for

20 min. The supernatant was incubated with Ni-NTA beads (Qiagen) in lysis buffer I. After washing in the same buffer, bound proteins were eluted with buffer F (20 mM Tris-Cl, pH7.4, 150 mM NaCl, 300 mM Imidazole). The His_6-SUMO tag was cleaved by SUMO protease (Ulp1) and the mixture was loaded onto a 1-ml HiTrap Heparin column (GE Healthcare) in buffer B. After washing with buffer B containing 0.5 M NaCl, the cGAS protein was eluted with 1.0 M NaCl in the same buffer.

GST-tagged cGAS was generated in pLys/BL21 using Gateway cloning according to manufacturer's instruction. After induction with 0.8 mM IPTG at 18ºC overnight, the bacteria were resuspended and sonicated in lysis buffer II [50 mM Tris-HCl, pH7.4, 100 mM NaCl, 10% glycerol, 0.5% NP40, 0.5 mM EDTA, 0.5 mM EGTA], and centrifuged at 100,000 x g for 20 min. The supernatant was mixed with glutathione Sepharose (GE Healthcare) and washed extensively with lysis buffer II. Bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-Cl, pH8.0, and dialysed against buffer G [20 mM Tris-Cl, pH. 7.4, 50mM NaCl, 10% Glycerol].

To express recombinant cGAS protein in mammalian cells, HEK293T was transfected with pcDNA3-Flag-h-cGAS for 36 hours. Cytosolic extracts were prepared from 20 plates (15-cm ID) of cultured cells by douncing in a hypotonic buffer [10mM Tris-Cl. pH7.4, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT and protease inhibitor cocktail (Roche)], and loaded onto a 5-ml HiTrap Heparin column in buffer B. After washing with 0.5M NaCl in buffer B, Flag-h-cGAS was eluted with 1.0M NaCl and then mixed with agarose beads coupled with anti-Flag (M2) antibody (Sigma). After washing in lysis buffer II, bound proteins were eluted with 0.2 mg/ml FLAG peptide in a buffer containing 50 mM Tris-Cl, pH8.0, and 0.02% CHAPS.

DNA Binding Assay

Recombinant GST-tagged cGAS was incubated with streptavidin UltraLink beads in the presence of ISD or biotin-ISD in lysis buffer II. After three washes in the same buffer, bound proteins were eluted by boiling in SDS sample buffer and detected by immunoblotting using a GST antibody (Covance). A biotin-RNA sequence

(ACGGAAAGACCCCGU) from 23S rRNA of DH5α was used as a negative control. To map the DNA binding domains of cGAS, Flag-tagged cGAS fragments were expressed in HEK293T cells by transient transfection. Cell lysates were prepared in lysis buffer II and incubated with streptavidin UltraLink beads as above, except that cGAS protein fragments precipitated by the beads were detected with a Flag antibody (M2).

Protein Identification by Mass Spectrometry

Fractions from the last step of each purification route were resolved by SDS-PAGE and then visualized by silver staining. For each purification route, one lane from the fraction containing the highest cGAS activity and two adjacent lanes from fractions containing low cGAS activity were chosen for mass spectrometry analyses. Each lane was cut into 10 slices of gels of approximately equal sizes, which were destained and then reduced in 20 mM DTT at 56°C for 30 min followed by alkylation in 55 mM iodoacetamide in the dark for 1 hr. Proteins in the gels were digested in situ with sequence grade trypsin (Promega) in 50 mM ammonium bicarbonate at 37°C overnight. Peptides were extracted sequentially with 5% formic acid (FA) /50% acetonitrile (ACN) and 0.1% FA /75% ACN, vaccum dried and then resuspended in 10 µl of 0.1% FA. 5 µl of the peptide sample was loaded via an autosampler (Thermofisher Scientific) onto a homemade analytical column (75 µm ID, 150 mm length) packed with C-18 resin (100 Å, 3 µm, MICHROM Bioresources). The peptides were then eluted with a 78 min gradient generated by a Dionex Ultimate 3000 nanoLC system (Thermofisher Scientific) as follows: 2–30% B in 68 min, 30–35% B in 4 min, 35–40% B in 2 min, 40–60% B in 3 min, and 60–80% B in 1 min (A = 0.1% FA; B = 0.1% FA/ 100% ACN). The eluted peptides were sprayed into to a Quadrupole-Orbitrap Hybrid mass spectrometer (Q-Exactive, ThermoFisher Scientific) equipped with a nano-electrospray ion source. MS/MS spectra were acquired in a data-dependent mode whereby the top 10 most abundant parent ions were subjected to further fragmentation by higher energy collision dissociation (HCD). For protein identification, database searches were performed on an in-house Mascot server (Matrix Science) against the IPI mouse database (v3.87). Carbamidomethylcysteine was set as a fixed modification and N-acetylation and methionine oxidation were set as variable modifications.

Quantitative Analysis of Mass Spectrometry Data

For label-free quantification of proteins identified in the fractions from the last step of each purification route, raw data were searched again using the Andromeda search engine and the MaxQuant software package (1.3.0.5) against the IPI mouse database (v3.87) (*3, 4*). The first search tolerance was set at 20 ppm and main search deviation at 6 ppm. The required minimum peptide length was six amino acids. The false discovery rate (FDR) at both peptide and protein levels was set to 0.01. The methods for comparative analysis of the amount of a protein in different fractions were defined manually and coupled to MaxQuant through the Experimental design file. To get protein candidates that correlated with the cGAS activity in different purification routes, MaxQuant output results were verified manually with a comprehensive consideration of peptide counts, sequence coverage and protein intensities. The Venn diagram showing proteins that overlapped among different purification routes was generated by the "VENNY" online server. Mass spectrometry and SRM quantification of cGAMP was described in Wu et al (*1*).

Immunostaining and confocal microscopy

Immunostaining with L929 cells stably expressing Flag-tagged m-cGAS was performed as described previously(*2*). Cells were transfected with Cy3-ISD for the indicated times and fixed in 4% formaldehyde in PBS for 15 min. Anti-Flag M2 (1:500, Sigma) and Alexa 488 conjugated goat anti-mouse IgG (1:500, Invitrogen) were used as the primary and secondary antibodies to detect Flag-cGAS. Nuclei were labeled by staining with DAPI in the mounting medium (Vectashield). Images of the cells were collected with a Zeiss LSM510 META laser scanning confocal microscopy (Carl Zeiss MicroImaging Inc).

Subcellular Fractionation

Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For fractionation of subcellular organelles, THP-1 cells were washed with cold PBS and lysed by douncing for 20 times in the hypotonic buffer. The homogenate was centrifuged at 500 x g for 5 min to remove cell debris and nuclei. The supernatant (S1) was centrifuged at 5,000 x g for 10 min to precipitate mitochondria and other heavy organelles (P5). The supernatant (S5) was further centrifuged at 17,000 x g for 10 min to generate S20 and P20. S20 was further centrifuged at 100,000 x g to obtain S100 and P100. Each pellet was resuspended in equal volumes of lysis buffer II for further analyses by immunoblotting.

Figure S1

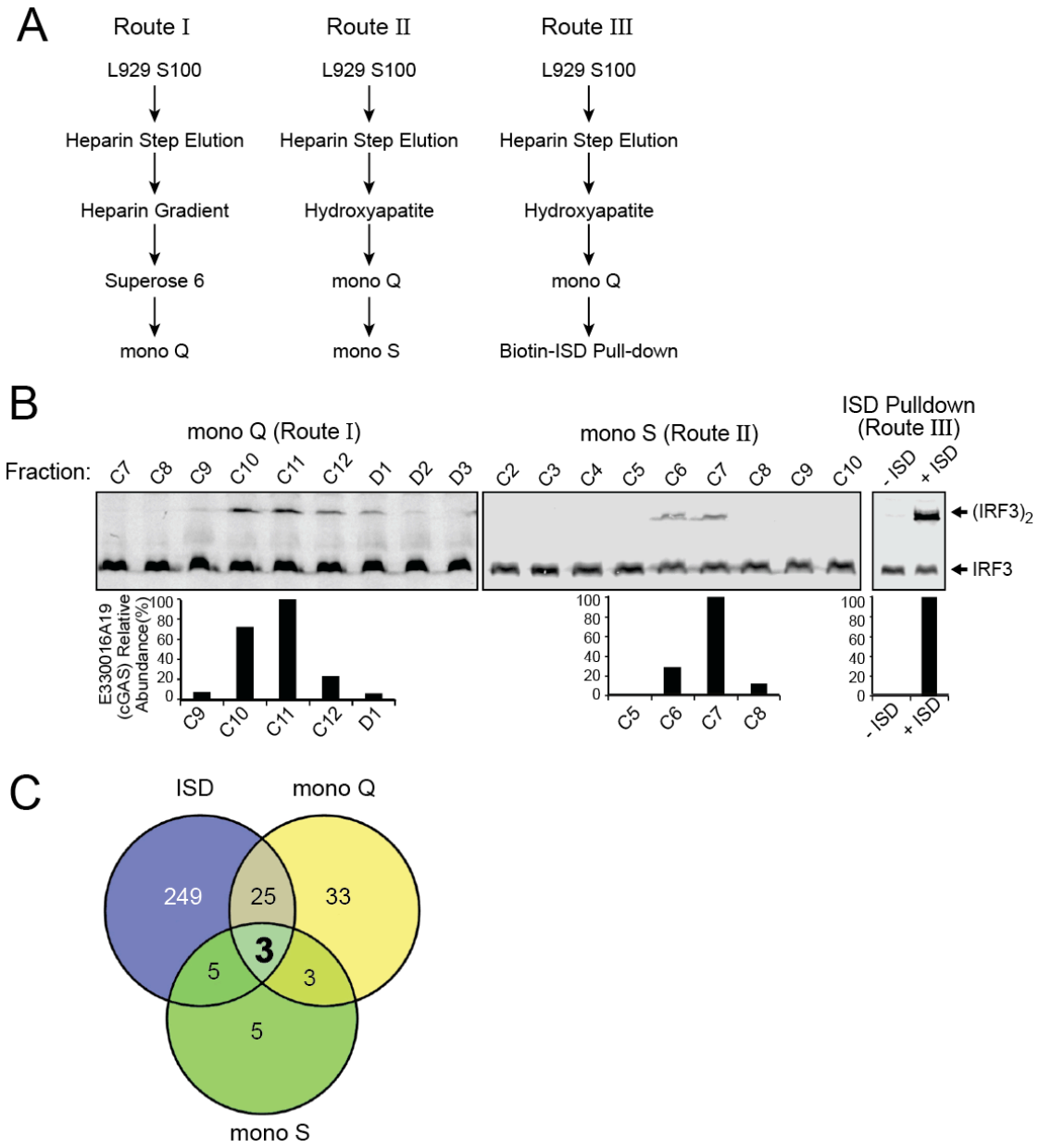

Figure S1. Purification of a cGAMP synthase (cGAS) and its identification by quantitative mass spectrometry. (**A**) Diagram of three different routes to purify endogenous cGAS activity from L929 cell extract. (**B**) Fractions from the last step of each purification route were tested for the activity to generate cGAMP, which was delivered to permeabilized Raw264.7 cells followed by measurement of IRF3 dimerization. The relative abundance of E330016A19 (m-cGAS) was estimated by label-free quantification of mass spectrometry data generated from 80 samples (gel slices). (**C**) Venn diagram depicting the number of proteins that overlapped among different purification routes based on quantitative mass spectrometry analyses.

Figure S2

A

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  1 MEDPRRRTTA PRAKKPSAKR APTQPSRTRA HAESCGPQRG ARSRRAERDG
 51 DTTEKPRAPG PRVHPARATE LTKDAQPSAM DAAGATARPA VRVPQQQAIL
101 DPELPAVREP QPPADPEARK VVRGPSHRRG ARSTGQPRAP RGSRKEPDKL
151 KKVLDKLRLK RKDISEAAET VNKVVERLLR RMQKRESEFK GVEQLNTGSY
201 YEHVKISAPN EFDVMFKLEV PRIELQEYYE TGAFYLVKFK RIPRGNPLSH
251 FLEGEVLSAT KMLSKFRKII KEEVKEIKDI DVSVEKEKPG SPAVTLLIRN
301 PEEISVDIIL ALESKGSWPI STKEGLPIQG WLGTKVRTNL RREPFYLVPK
351 NAKDGNSFQG ETWRLSFSHT EKYILNNHGI EKTCCESSGA KCCRKECLKL
401 MKYLLEQLKK EFQELDAFCS YHVKTAIFHM WTQDPQDSQW DPRNLSSCFD
451 KLLAFFLECL RTEKLDHYFI PKFNLFSQEL IDRKSKEFLS KKIEYERNNG
501 FPIFDKL
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B

	human	mouse	rat	dog	chicken	lizard	Frog	Zebrafish
human	100							
mouse	56	100						
rat	55	88	100					
dog	60	51	51	100				
chicken	46	45	42	44	100			
lizard	40	42	41	37	43	100		
Frog	28	26	26	21	27	26	100	
Zebrafish	32	35	33	29	33	30	26	100

C

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human          157 ----------DAA-------PGASKLRAVLEKLKLSRDDISTAAGMVKGVVDHLLLRLKC--DSAFRGVG 207
mouse          138 ----------RAPRGSR---KEPDKLKKVLDKLRLKRKDISEAAETVNKVVERLLRRMQKR-ESEFKGVE 193
Rat            141 ----------RASQGSR---KEPDKLKKVLDKLRLKRKEISAAAETVNKVVDQLLRRMQRR-ESEFKGVE 196
dog            145 ----------RPA-------PGAWKPRLVLEKLRLRRQEISEAAEVVNRVVDYLSRRLQSY-ESEFRGVS 196
chicken        117 ----------SRA-------AGGLRLREVLSQLSLGRQDVSEASGLVNHVVSHLIQAVRGR-DGSFSSIR 168
lizard         206 ----------GAA-------PASPALRPVLERLRLGREARSEAADQVNQLRDRLVRAVKK--VPGFKTVE 256
zebrafish      218 ----------VKD-------ALGDVLKATLDKLTIKKSERSKASRCVNEITEKVIAHLKQD-TTWCADIE 269
Consensus_ss:                        hhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhh        ee

                     **          * *

human          208 LLNTGSYYEHVKISAPNEFDVMFKLEVPRIQLEEY---SNTRAYYFVKFKRNPKENPLSQFLE-GEILSA 273
mouse          194 QLNTGSYYEHVKISAPNEFDVMFKLEVPRIELQEY---YETGAFYLVKFKRIPRGNPLSHFLE-GEVLSA 259
Rat            197 QLNTGSYYEHVKISAPNEFDVMFKLEVPRIELEEY---YETGAFYRVKFKRIPRGNPLSHFLE-GEVLSA 262
dog            197 LLRTGSYYEHVKISAPNEFDIMFKLEVPRIQLEEY---CKTGAYYFVKFKRNPKGNPLSQFLE-DEKLSA 262
chicken        169 RLGAGSYYEHVKISEPNEFDIMLVMPVTRLQLDES---DDTGAYYYLTFKRNPKEKYLNRFLDEDGKLSA 235
lizard         257 VFGAGSYYEGAKICSPNEFDIMLKIPEVRLELESCEVVSSSGAFHYVKLKRDPANKELREFCEDNERLSS 326
zebrafish      270 RLRTGSYYENLKICEPDEFDVMLTVPVERVDIQNF---DEAGAFYSIALKRHPNKHPLDKFLNEDKTIQA 336
Consensus_ss:      e       eee     eeeeeee                 eeeee        hhhhhhh      h

                                                            *
human          274 SKMLSKFRKIIKEEINDIKD--TDVIMKRKRGGSPAVTLLISE---KISVDITLALESK-SSWPASTQEG 337
mouse          260 TKMLSKFRKIIKEEVKEIKD--IDVSVEKEKPGSPAVTLLIRNPE-EISVDIILALESK-GSWPISTKEG 325
Rat            263 TKVLSKFRELIKEEVKEIKD--TDVTVEEEKPGSPAVTLLIRNPE-EISVDIILALESK-GSWPISTKGG 328
dog            263 SKMLSKFRKIIKEEIKNIQA--TDIIMEKKKRGSPAITLLIRKPK-EISVDIILALESK-SSWPASTQEG 328
chicken        236 FKMLEDLRRIIKEELKHIKN--VEVTVKRKKRGSPAITLLIKKPPAEISVDIILALEVQ-QSWPPSTQDG 302
lizard         327 SRMLLGLRNIIMDEVKKMAD--MNVSVEKKRPGSPAVTILIGSPPSVISVDIILALEFHHPKWCSSTENG 394
zebrafish      337 SEMLSEFRDGVKKAVEKATDLPYKIKIQRKKPKCPAVTLEVTEGRKNISVDFVLGLKVHRASWPELTKDG 406
Consensus_ss:      hhhhhhhhhhhhhhhhh       eeeeeee     eeeeeee     eeeeeeeeeeee      hhh

human          338 LRIQNWLSAKVRKQLRLKPFYLVPKHAK-----EGNGFQ-----EETWRLSFSHIEKEILNNHGKSKTCC 397
mouse          326 LPIQGWLGTKVRTNLRREPFYLVPKNAK-----DGNSFQ-----GETWRLSFSHTEKYILNNHGIEKTCC 385
Rat            329 LPIQDWLGTKVRTNLRREPFYLVPKNAK-----DGNRFQ-----GETWRLSFSHTEKYILNNHGIGKTCC 388
dog            329 LPISHWLGTKVKTNLRRQPFYLVPKHAK-----EGNYFQANRPLQPQLQLVPLVVYFGLM---------- 383
chicken        303 LNIECWLGRKVRREFRYKPIYLVAKQNK-----KEKLPR-----GNTWRLSFSHIEKAMLNNHGSIKTCC 362
lizard         395 LGIEEWMGTKVKKSLRSEPLYLVTKNVK-----DGKCFI-----ENTWRLSFSNIEKMLLQNHGQTKTCC 454
zebrafish      407 FRVEHWLGRKVKSTMKRQPFYLVPKYEGIGNAEHDGVVA-----RDSWRISFSHIEKEILKSHGHTKTCC 471
Consensus_ss:          hhhhhhhh                              hhhhhhhhhhhhhhhh       hh

human          398 ENKEEKCCRKDCLKLMKYLLEQLKERFKDKKHLDKFSSYHVKTAFFHVCTQNPQDSQWDRKDLGLCFDNC 467
mouse          386 ESSGAKCCRKECLKLMKYLLEQLKKE---FQELDAFCSYHVKTAIFHMWTQDPQDSQWDPRNLSSCFDKL 452
Rat            389 ESSGAKCCRKECLKLMKYLLEQLKRE---FQELDAFCSYHVKTAIFHMWTKDPQDSQWDPRNLSTCFDKF 455
dog            384 ---VISVFRKDCLKLMKYLLEQLKTKFGNRKELDKFCSYHVKTAFFHVCTQDPQDSQWQAKDLELCFDNC 450
chicken        363 ESDGVKCCRKDCLKLLKYLLERLKMKH--AKELEKFCSYHVKTAFFHSCVTWPSDTDWQHENLEHCFQKY 430
lizard         455 EIKGEKCCRKDCLKLLKYLLLQLKTKHENRNQFEKFCSYHVKTAFFHACVKWPKDSDWLLANLDECFNRL 524
zebrafish      472 EGREQKCCRKECLKLLKYLLQQLKNDESKSNKMSSFCSYHAKTTLLQACASRGTDIEWAYSELANCFQQL 541
Consensus_ss:      hhhhhhhhhhhhhhhhhhhhhhh          hhhhhhhhhhhhhh        hhhhhhhhhhh

human          468 VTYFLQCLRTEKLENYFIPEFNLFSSNLIDKRSKEFLTKQIEYERNNEFPVFDEF  522
mouse          453 LAFFLECLRTEKLDHYFIPKFNLFSQELIDRKSKEFLSKKIEYERNNGFPIFDKL  507
Rat            456 LTFFLECLRTEKLDHYFIPKFNLFSQELIDKKSKEFLSEKIEYERNNGFPIFNKF  510
dog            451 VTYFLHCLKKEQLNHYFIPEFNLFSRDQIDKISKEFLLQQIEYERNNGFPVFHEF  505
chicken        431 LEYFLRCLQDSQLPHFFIPQYNLLSL--DDKASNDFLLGQINYQLNNGFPVFHQV  483
lizard         525 LNYFLDCLKEAKLPHFFIPGFNLFSEERIDRTKRNALVKTIEKEINNRFRIFFN-  578
zebrafish      542 LEDFVKHLKNHQLPNFFIPSHNLLHH--VSTSNCDFLAKEIEFQLNNTFPIFS--  592
Consensus_ss:      hhhhhhhhh                  hhhhhhhhhhhhhhhhh
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Figure S2. Evolutionary conservation of cGAS sequences in vertebrates. (**A**) Amino acid sequence of mouse cGAS protein (E330016A19). Peptides identified by mass spectrometry are shown in red. (**B**) Identity scores between amino acid sequences of cGAS from pairs of vertebrate species. (**C**) Multiple sequence alignment of the C-terminal conserved regions of cGAS proteins from various species using the *PROMALS3D* program. Identical amino acids are indicated in red and conserved amino acids in yellow. Predicted secondary structure elements are indicated below the alignment as alpha helices (*h*) and beta strands (*e*). Conserved active site residues of NTase superfamily are marked with an asterisk (*).

Figure S3

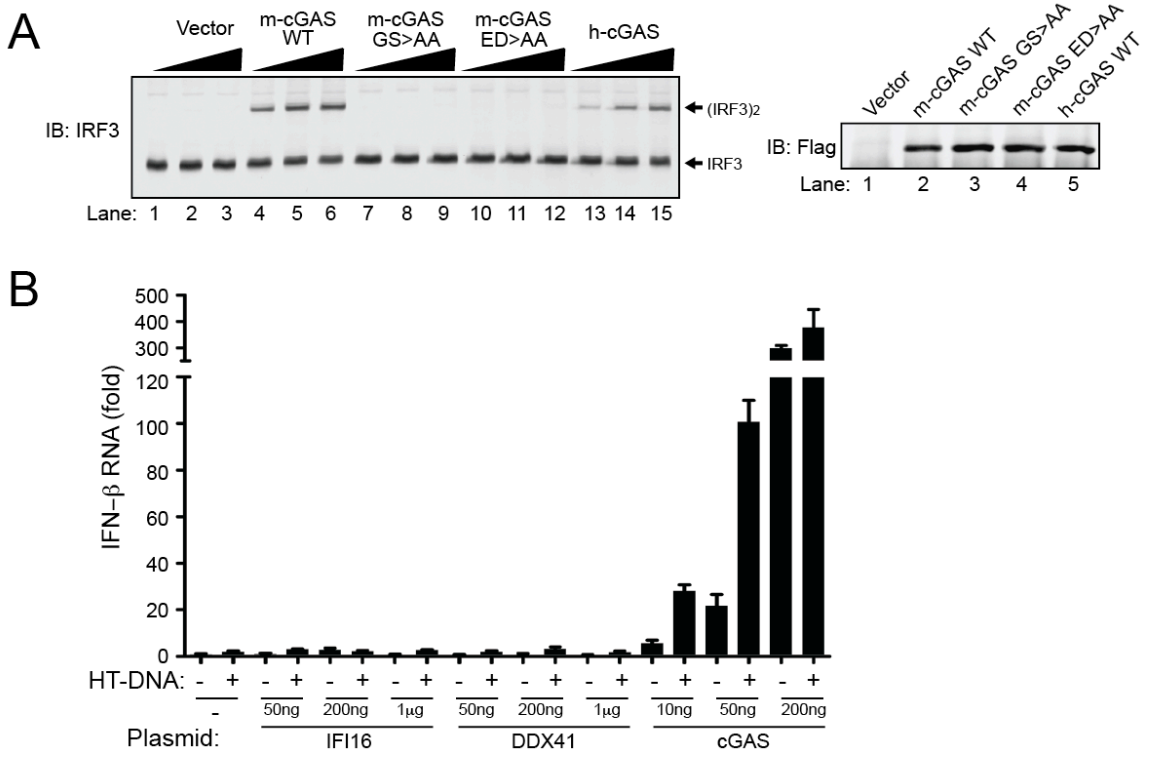

Figure S3. cGAS activates IRF3 and induces IFNβ. (**A**) Indicated proteins were expressed in HEK293T cells and affinity purified using Flag antibody (M2)-agarose. The proteins were incubated with ATP and GTP in the presence of HT-DNA, and the synthesis of cGAMP was assessed by its ability to induce IRF3 dimerization in Raw264.7 cells (left). Aliquots of the cGAS proteins were immunoblotted with a Flag antibody (right). (**B**) Indicated amounts of the expression plasmids were transfected with or without HT-DNA (3 μg) into HEK293T-STING cells, followed by measurement of IFNβ RNA by q-RT-PCR.

Figure S4

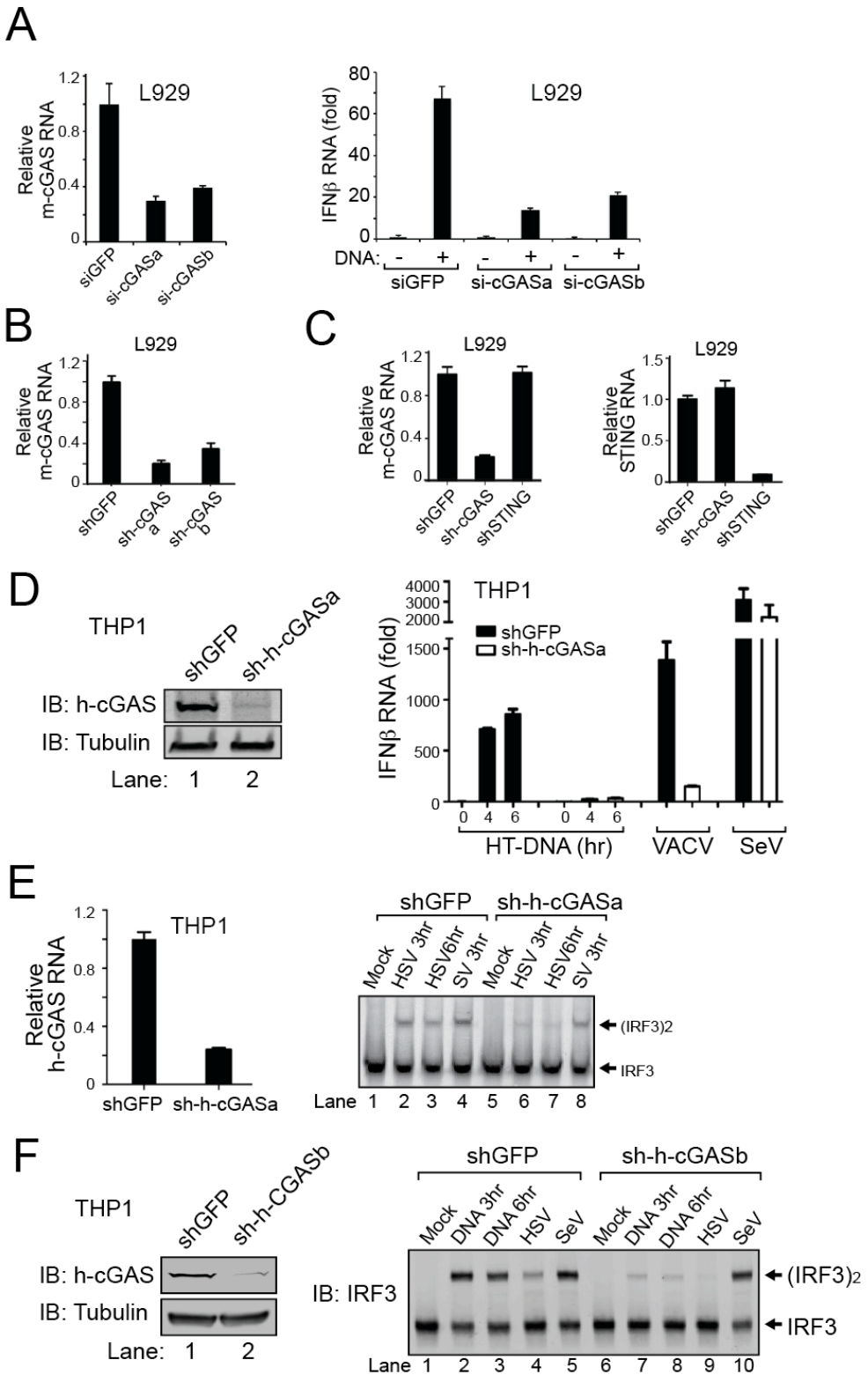
A
L929
Relative m-cGAS RNA
siGFP
si-cGASa
si-cGASb
L929
IFNβ RNA (fold)
DNA:
siGFP
si-cGASa
si-cGASb
B
L929
Relative m-cGAS RNA
shGFP
sh-cGAS a
sh-cGAS b
C
L929
Relative m-cGAS RNA
shGFP
sh-cGAS
shSTING
L929
Relative STING RNA
shGFP
sh-cGAS
shSTING
D
THP1
shGFP
sh-h-cGASa
IB: h-cGAS
IB: Tubulin
Lane: 1 2
THP1
shGFP
sh-h-cGASa
IFNβ RNA (fold)
HT-DNA (hr)
VACV
SeV
E
THP1
Relative h-cGAS RNA
shGFP
sh-h-cGASa
shGFP
sh-h-cGASa
Mock
HSV 3hr
HSV6hr
SV 3hr
(IRF3)2
IRF3
Lane 1 2 3 4 5 6 7 8
F
THP1
shGFP
sh-h-CGASb
IB: h-cGAS
IB: Tubulin
Lane: 1 2
shGFP
sh-h-cGASb
Mock
DNA 3hr
DNA 6hr
HSV
SeV
IB: IRF3
(IRF3)2
IRF3
Lane 1 2 3 4 5 6 7 8 9 10

Figure S4. cGAS is required for IRF3 activation and IFNβ induction. (**A**) L929 cells were transfected with two different pairs of siRNA oligos targeting distinct regions of mouse cGAS. As a control, siRNA against GFP was also transfected into the cells. 60 hr after transfection, cells were transfected with HT-DNA for another 8 hr and then IFNβ and m-cGAS RNA was measured by q-RT-PCR. (**B**) q-RT-PCR analyses of m-cGAS RNA in L929 cell lines stably expressing shRNA targeting GFP (control) or two different regions of m-cGAS. (**C**) RNA from L929 cells stably expressing a shRNA against GFP, m-cGAS or STING was measured by q-RT-PCR. (**D**) THP1 cells stably expressing a shRNA against GFP or human cGAS were transfected with HT-DNA or infected with Vaccinia virus (VACV) or SeV. 6 hr later, IFNβ RNA was measured by q-RT-PCR. The knockdown of h-cGAS in the cells was assessed by immunoblotting. (**E** and **F**) THP1 cells stably expressing two different shRNAs targeting distinct regions of h-cGAS were analyzed by q-RT-PCR (E) or immunoblotting (F) to determine the knockdown efficiency. These cells were infected with HSV1 or SeV for 6 hr (E and F) or transfected with HT-DNA for different lengths of time (F), then IRF3 dimerization was analyzed by native gel electrophoresis.

Figure S5

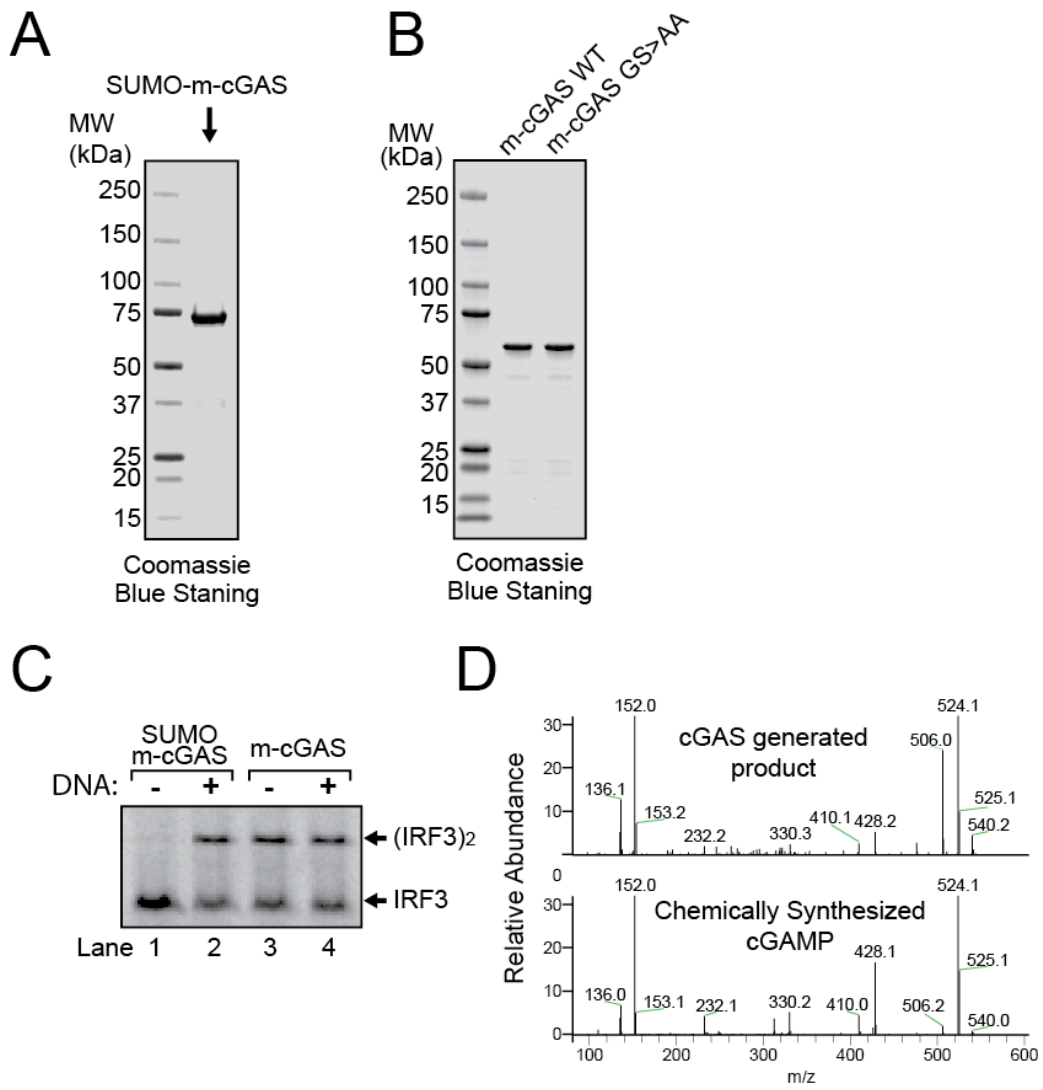

Figure S5. Purification and catalytic activity of recombinant cGAS protein. (**A**) Coomassie blue staining of Sumo-m-cGAS expressed and purified from *E coli*. (**B**) Coomassie blue staining of wild-type and GS>AA (G198A/S199A) mutant of m-cGAS expressed and purified from *E coli*. The Sumo tag was removed by a Sumo protease (Ulp1). (**C**) Purified Sumo-m-cGAS or m-cGAS lacking the Sumo tag was incubated with ATP and GTP in the presence or absence of HT-DNA, then the reaction mixtures were heated to prepare heat-resistant supernatants, which were delivered to permeabilized Raw264.7 cells to measure IRF3 activation. (**D**) Comparison of the MS/MS spectra of cGAMP produced by purified m-cGAS and chemically synthesized cGAMP.

Figure S6

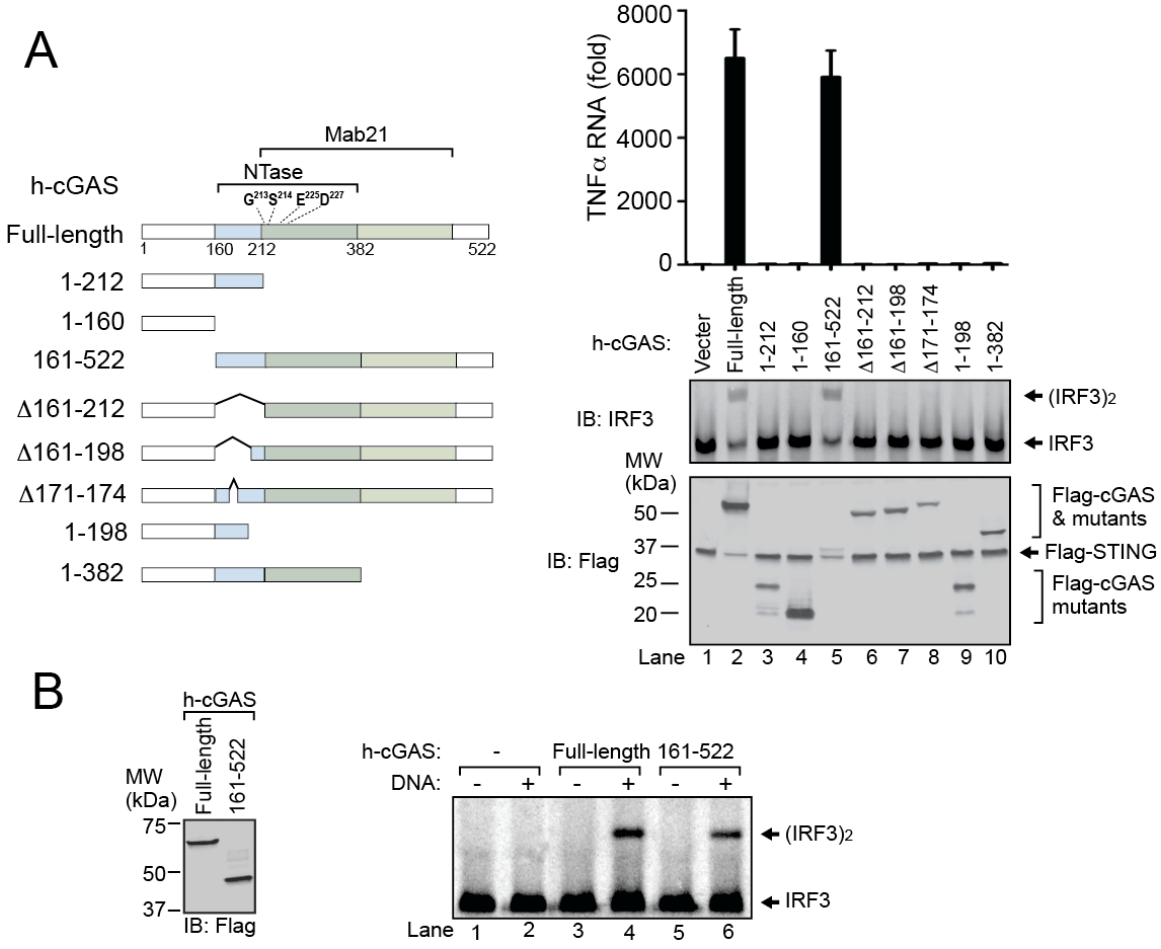

Figure S6. Dissection of cGAS functional domains. (**A**) Expression vectors encoding Flag-tagged full-length h-cGAS and various deletion mutants as illustrated (left) were transfected into HEK293T-STING cells. 24 hr after transfection, TNFα RNA was measured by q-RT-PCR. Aliquots of the cell extracts were resolved by native gel electrophoresis and SDS-PAGE, followed by immunoblotting with antibodies against IRF3 and Flag, respectively. (**B**) Flag-tagged full-length h-cGAS and the fragment containing residues 161-522 were expressed in HEK293T cells and then affinity purified. These proteins were incubated with ATP and GTP in the presence or absence of HT-DNA, and the reaction products were analyzed for the presence of cGAMP activity by measuring IRF3 dimerization in Raw264.7 cells.

Figure S7

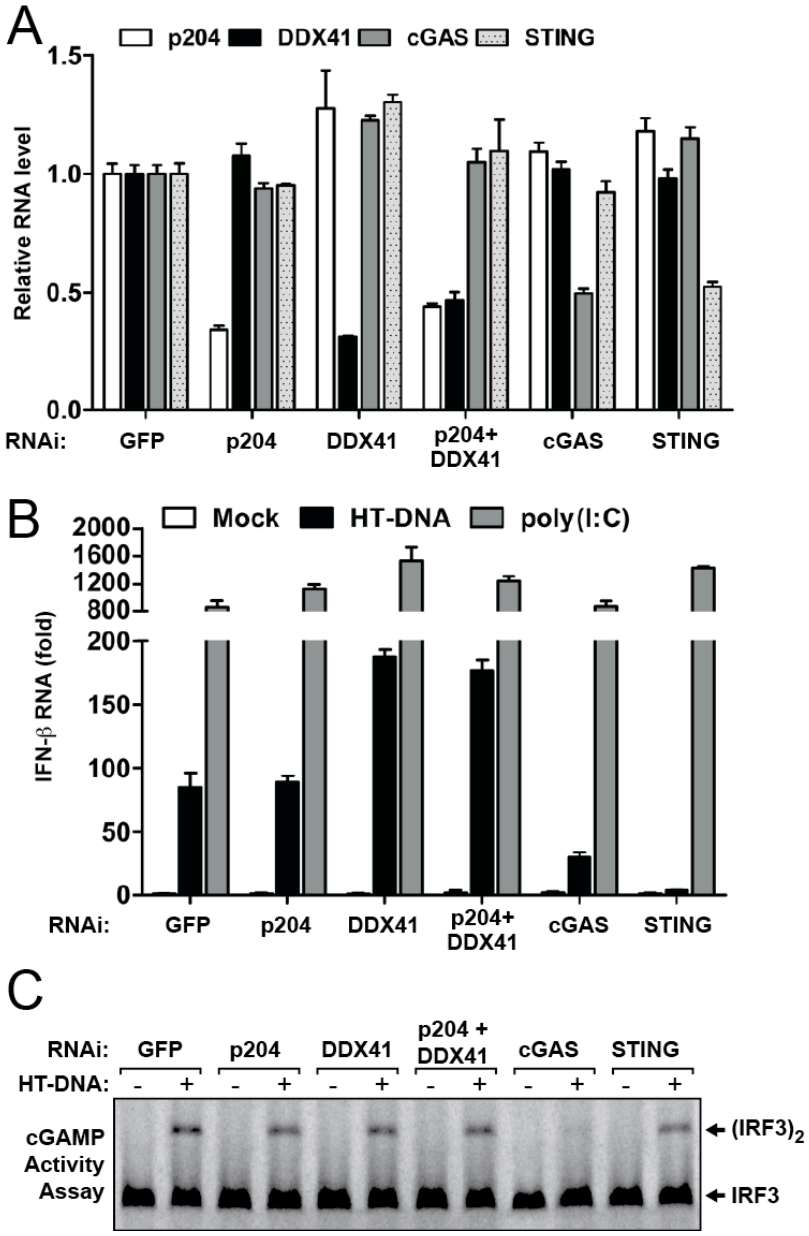

Figure S7. Knockdown of cGAS, but not DDX41 or p204, inhibited the generation of cGAMP activity in DNA-transfected cells. siRNA against the indicated genes was transfected into L929 cells followed by transfection of HT-DNA. The RNA levels of each RNAi target (**A**) and IFNβ (**B**) were measured by q-RT-PCR. Aliquots of the cell lysates were heated to prepare heat-resistant supernatants, which were delivered into permeabilized Raw264.7 cells to measure the activity of cGAMP, as assessed by the stimulation of IRF3 dimerization (**C**). As expected, knockdown of cGAS, but not STING, inhibited the generation of cGAMP activity in the cells.

References

1. J. Wu *et al*., Cyclic-GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science*, (2012).
2. Y. Tanaka, Z. J. Chen, STING Specifies IRF3 Phosphorylation by TBK1 in the Cytosolic DNA Signaling Pathway. *Sci Signal* **5**, ra20 (2012).
3. J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**, 1367 (Dec, 2008).
4. J. Cox *et al*., Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. *Journal of Proteome Research* **10**, 1794 (Apr, 2011).