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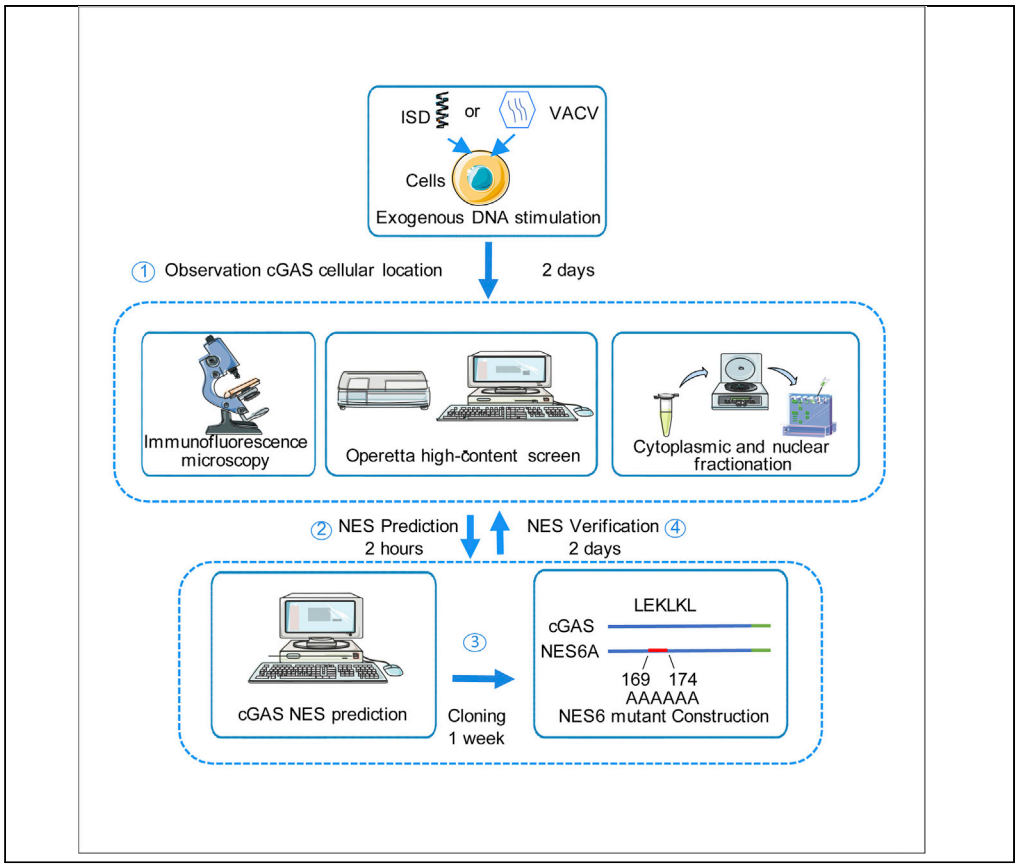
Protocol for nuclear export signal characterization of cGAS in mammalian cells



The cyclic GMP-AMP synthase (cGAS) is the principal DNA sensor, which binds DNA and synthesizes cGAMP, and triggers the type I interferon production. We used ISD45 or inactivated VACV to stimulate cGAS and monitored cellular localization by immunofluorescence microscopy, Operetta high-content screening, and cytoplasmic/nuclear fractionation. LocNES server was used to predict cGAS nuclear export signal sequence and characterized the function by mutagenesis. This protocol provides a prototype of cGAS subcellular distribution or the identification of NES in other proteins.

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Highlights

LocNES is used to predict cGAS nuclear export signal

Validation of cGAS nuclear export signal

cGAS localization detected by microscopy and cell fractionation

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Protocol for nuclear export signal characterization of cGAS in mammalian cells

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SUMMARY

The cyclic GMP-AMP synthase (cGAS) is the principal DNA sensor, which binds DNA and triggers the type I interferon production. We used ISD45 or inactivated Vaccinia Virus (VACV) to stimulate cGAS and monitored cellular localization by immunofluorescence microscopy, Operetta high-content screening, and cytoplasmic/nuclear fractionation. LocNES server was used to predict cGAS nuclear export signal (NES) sequence and characterized the function by mutagenesis. This protocol provides a prototype of cGAS subcellular distribution or the identification of NES in other proteins.

For complete details on the use and execution of this protocol, please refer to Sun et al. Sun et al. (2021)

BEFORE YOU BEGIN

Cell preparation

Timing: 2 days

1. The HeLa cell line was purchased from ATCC (catalog number: CCL-2). HeLa cells were maintained in DMEM medium (catalog number A4192101, Gibco). 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin were added in medium. Cells were cultured at 37°C with 5% CO_2.

⚠ CRITICAL: Constantly monitor cell growth state to ensure consistent experimental conditions.

2. Seed HeLa cells into a glass bottom cell culture dish (NEST, 801001, ϕ20 mm) (about 1.5×10^5cells/dish). Culture cells for 16–18 h to reach 60%–70% confluency, and then transfect cells with ISD45 DNA (details are described in section "ISD and plasmid transfection") or stimulate cells with heat inactivated Vaccinia virus of MOI=1 for 4 h.

⚠ CRITICAL: A suitable cell density (60%–70% confluency) is critical to ensure a high transfection efficiency. In order to obtain the suitable cell density, we seed $5*10^5$ cells in 6 wells plate and $1.5*10^5$ cells in confocal dishes.

Virus preparation

Timing: 30 min

3. Vaccinia virus (VACV, Tiantan strain) was propagated in Vero cells (Sun et al., 2021). Viruses were inactivated by incubation at 56°C for 30 min.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
ISD45 (5′- TACAGATCTACTAGTGATCTATGACTGATCTGTACA TGATCTACA-3′)	InvivoGen	tlrl-isdn
P1F (5′-AATTCGCCACCATGCCCAAGAAGAAGAGGAAGGTGC GG-3′)	Home designed and synthesized	N/A
P1R (5′-GATCCCGCACCTTCCTCTTCTTCTTGGGCATGGTG GCG-3′)	Home designed and synthesized	N/A
P2F (5′-AATTCGCCACCATGTTGGAGAAGTTGAAGCTCCCCAA GAAGAAGAGGAAGGTGCGG-3′)	Home designed and synthesized	N/A
P2R (5′-GATCCCGCACCTTCCTCTTCTTCTTGGGGAGC TTCAACTTCTCCAACATGGTGGCG-3′)	Home designed and synthesized	N/A
cGASE-F (5′-GGCGAATTCGCCACCATGCAGCCTTGGC ACGGAAAG-3′)	Home designed and synthesized	N/A
cGASE-R (5′-TGGGAATCCCGAAATTCATCAAAAACTGG AAACTC-3′)	Home designed and synthesized	N/A
NES6A-R (5′-TCGAAGCTCCGGGCGGTTGCGGCGGC GGCGGCGGCCAGCCGCGATGATATCTCC-3′)	Home designed and synthesized	N/A
NES6A-F (5′-GGAGATATCATCGCGGCTGGCCGCCGCC GCCGCCGCAACCGCCCGGAGCTTCGA-3′)	Home designed and synthesized	N/A
Antibodies		
Anti-Lamin A antibody	Sigma-Aldrich	Cat# L1293, RRID: AB_532254
Anti-β-Actin antibody	Sigma-Aldrich	Cat# A1978, RRID: AB_476692
Anti-beta-Tubulin	ProteinTech	Cat# 10094-1AP,RRID: AB_2210695
Anti-cGAS antibody	Cell Signaling Technology	Cat# 15102, RRID: AB_2732795
IRDye 800CW Goat anti-Mouse IgG Antibody	LI-COR Biosciences	Cat# 926-32210, RRID: AB_621842
IRDye 800CW Goat anti-Rabbit IgG Antibody	LI-COR Biosciences	Cat# 926-32211, RRID: AB_621843
Alexa Fluor Plus 488	Invitrogen	A32766
Bacterial and virus strains		
DH5α	Transgene	CD201
VACV (Tiantan strain)	Li Ruan, China CDC	N/A
Experimental models: cell lines		
HeLa	ATCC	CCL-2
Recombinant DNA		
pEGFP-N1	Clontech	6085-1
pEGFP-N1-cGAS	Home saved	N/A
Chemicals, peptides, and recombinant proteins		
PEI	Sigma-Aldrich	408727
PBS	Gibco	20012027
Trypsin-EDTA	Gibco	25200-056
DMEM basic (1×)	Gibco	A4192101
Protease inhibitors	Sigma-Aldrich	S8830
Nonidet P-40	Solarbio	N8030
FBS	Gibco	10091
SDS	Solarbio	S8010
Tris	Sigma-Aldrich	V900483

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Na_3VO_4	Sigma-Aldrich	S6508
NaF	Sigma-Aldrich	201154
Glycine	Sigma-Aldrich	V900144
DAPI	Sigma-Aldrich	324355
DTT	Sigma-Aldrich	D9779
BSA	Solarbio	A8010
Triton X-100	Solarbio	T8200
Tween-20	Solarbio	T8220
Paraformaldehyde	Solarbio	P1110
EDTA	Solarbio	E8040
KOD plus	Toyobo	KMM-201
T4 ligase	NEB	M0202S
T4 PNK	NEB	M0201S
EcoRI-HF	NEB	R3101S
BamH1-HF	NEB	R3136S
Software and algorithms		
GraphPad	GraphPad Prism 7	N/A
ImageJ	NIH	N/A
ZEN microscope software	Zeiss	N/A
Other		
Operetta High-Content Screen System	PerkinElmer	N/A
Elyra 7 Lattice SIM	Zeiss	N/A
LiCor Odyssey instrument	LI-COR Biosciences	N/A
Nitrocellulose membranes	Whatman	N/A
CellCarrier-96 plate	PerkinElmer	6005550
Cell culture dish (φ20 mm)	NEST	801001

MATERIALS AND EQUIPMENT

Buffer A (stocks can be kept at 4°C for 6 months)	
Reagents	Final concentration
Tris-HCl	20 mM(pH7.6)
EDTA	0.1 mM
$MgCl_2.6H_2O$	2 mM
NaF	0.5 mM
Na_3VO_4	0.5 mM

Buffer B (stocks can be kept at 4°C for 6 months)	
Reagents	Final concentration
HEPES	20 mM (pH7.9)
NaCl	400 mM
Glycerol	25%(vol/vol)
EDTA	1 mM
NaF	0.5 mM
Sodium deoxycholate	0.5 mM
DTT	0.5 mM

RIPA (stocks can be kept at 4°C for 6 months)	
Reagents	Final concentration
NP-40	1% (vol/vol)
Tris-HCl	50 mM (pH7.4)
NaCl	150 mM
Na_3VO_4	0.25%
protease inhibitors	1×

10× Running buffer (stocks can be kept at 25°C for 6 months)		
Reagents	Final concentration	Amount (for a 1 L stock)
Tris-HCl	250 mM	30.3 g
Glycine	2 M	144 g
SDS	1%	10 g
ddH_2O	To 1 L	

Transfer buffer (stocks can be kept at 25°C for 6 months)		
Reagents	Final concentration	Amount (for a 1 L stock)
Tris-HCl	250 mM	30.3 g
Glycine	2 M	144 g
SDS	1%	10 g
Methanol	20%	200 mL
ddH_2O	To 1 L	

PBST (stocks can be kept at 25°C for 6 months)		
Reagents	Final concentration	Amount (for a 1 L stock)
PBS	0.5%	1L
Tween 20		500 μL

Permeabilization buffer (stocks can be kept at 25°C for 6 months)		
Reagents	Final concentration	Amount (for a 1 L stock)
PBS	0.3%	1L
Triton ×100		300 μL

Blocking buffer (compound when it is in need and stock in 4°C for 1 day)		
Reagents	Final concentration	Amount (for a 50 mL stock)
PBS	5%	50 mL
BSA		2.5 g

STEP-BY-STEP METHOD DETAILS

Exogenous DNA stimulation

Timing: 6 h

1. ISD transfection:
   a. Replace culture medium with 1 mL FBS-free DMEM 1 h before transfection.
   b. 1 μg/dish ISD was diluted in 300 μL FBS-free DMEM medium, and 2 μL PEI (ISD/PEI for 1:2) was added in another 300 μL FBS-free DMEM medium. Keep two mixtures at 25°C for 5 min.

c. Mix them with pipette, and leave the reaction at 25°C for 20 min. ISD or plasmid/PEI 600 μL mixture was used to replace DMEM medium in dishes, cells were cultured for 4 h at 37°C.
d. The DNA mixture was replaced with 1 mL complete DMEM medium and cultured at 37°C.

⚠ CRITICAL: The PEI/DNA ratio is important for high transfection efficiency. Too much (above 3:1) PEI is cytotoxic.

⚠ CRITICAL: Replacing the medium or DNA/PEI mixture should be done quickly, to avoid cells getting dry.

Alternatives: PEI was used for plasmid transfection. Elyra 7 Lattice SIM and Operetta High-Content Screen system were used to detect subcellular distribution of fluorescence signal. An equivalent transfection method (such as lipo2000 or lipo3000) and imaging system can be used (such as Leica STELLARIS 5).The nucleus and cytoplasm fractionation assay can also be performed using Subcellular Protein Fractionation Kit for Cultured Cells sold by Thermofisher (Cat# 78840).

2. VACV stimulation

Inactivated VACV (MOI=1) was added to HeLa cells with complete DMEM medium, and incubated for 4 h at 37°C in an incubator.

Detection of the subcellular localization of cGAS in exogenous DNA stimulation

Timing: 1 day for step 3

Timing: 1 day for step 4

Timing: 1 day for step 5

3. Detection of cGAS subcellular localization by Immunofluorescence microscopy
a. Seed cells in cell culture dish (ϕ20 mm) at approximately 30% confluency (1.5×10^5) post transfection or stimulation 18–20 h.
b. After 4 h stimulation with 1 μg ISD or inactivated VACV (MOI=1) as described above, discard culture medium, wash cells with sterile 1× phosphate-buffered saline (PBS) (1 mL/dish), fix cells with 4% paraformaldehyde (PFA) (in 1× PBS)for 10 min at 25°C.
c. Discard PFA, add 1 mL permeabilization buffer containing 0.3% Triton X-100 at 25°C for 10 min.

⚠ CRITICAL: Be precise on the time of permeabilization to avoid cell lysis.
d. Incubate cells in blocking buffer (5% BSA in PBS) for 1 h at 25°C, shake gently to best block non-specific protein-protein interaction.

⚠ CRITICAL: Time of blocking should not be too long (above 2 h) at 25°C. Place the plate at 4°C if blocking is more than 2 h.
e. Dilute the anti-cGAS antibody in 1 mL blocking buffer in a 1:1000 dilution. Incubate cells with this primary antibody for 2 h at 25°C or more than 12 h at 4°C.

⚠ CRITICAL: Dilution of primary antibody needs to be optimized based on the source of the antibodies.
f. Discard antibody solution, and wash cells 5 min/time for three times with PBST buffer on a horizontal rotator.

g. Dilute secondary antibody (conjugated Alexa Fluor (488)) with PBS (1:1000), incubate 1 mL diluted secondary antibody with cells for 1 h in dark at 25°C.

⚠ CRITICAL: For secondary antibody incubation, keep samples in dark until the experiment is over to avoid fluorescence quenching.

h. Discard antibody solution, wash cells once with 1 mL PBST for 5 min on horizontal rotator.

i. Dilute DAPI in PBS at 5 μg/mL and incubate 1 mL/well with cells in dark for 10 min at 25°C.

⚠ CRITICAL: Incubate time of DAPI should not exceed 15 min, to avoid high background fluorescence.

j. Wash cells with PBST twice with 5 min each time on a horizontal rotator.

k. Store the stained cells in 1 mL PBS at 4°C until examination with confocal microscope. Storage time should not exceed 48 h

l. Confocal images were acquired at 25°Cusing Elyra 7 Lattice SIM (ZEISS) mounted on an inverted microscope with an oil immersion 63×/numerical aperture 1.4 objective lens.

m. Determine cGAS localization in different sub-cellular compartments. Six different microscopic fields were counted by eye respectively. Cell counts were normalized in term of percentage. Graph was showed mean ± SEM (n=3 independent experiments) (Figures 1A and 1C).

⚠ CRITICAL: Avoid cell death in all the steps, which results in high fluorescence background.

Alternatives: cGAS subcellular distribution can also be evaluated using Fiji, a popular open source software.

4. Detection of cGAS subcellular localization by Operetta high-content screen (Dull et al., 2013)

a. Cell preparation

Seed HeLa cells at 10,000 per well in a CellCarrier-96 plate (6005550, PerkinElmer). 20 h later, 20 ng/well ISD was transfected or inactivated VACV (MOI=1) was added for a 4-h stimulation.

⚠ CRITICAL: Before plating cells into CellCarrier-96 plates, make sure to trypsinize cells thoroughly to obtain single cell suspension.

b. After exogenous DNA stimulation, fix cells with 100 μL/well 4% PFA with PBS at 25°C for 15 min.

c. Permeabilize cells by 100 μL/well 0.3% Triton-X100 for 15 min at 25°C.

d. Blocking cells with 100 μL/well 5% BSA in PBS for 1 h at25°C on a horizontal rotator.

e. Incubate cells with 50 μL/well anti-cGAS antibody diluted in 5% BSA/PBS (1:1000) on a horizontal rotator for 1 h at 25°C

f. Wash the cells with 100 μL/well PBST for 5 min, and repeat 3 times.

g. Incubate cells with 50 μL/well goat anti-Rabbit Alexa Fluor 488 antibody at 1:1000 (in PBS) dilution for 1 h at 25°C on horizontal rotator.

h. Wash cells with 1×PBST for 5 min on horizontal rotator.

i. Stain nuclei by DAPI (5 μg/mL) for 10 min in dark at 25°C.

j. Wash cells twice with 1× PBST 5 min/time on a horizontal rotator, store cells in 100 μL/well PBS.

k. Plates were then scanned, and images were collected using the Operetta HTS imaging system (PerkinElmer) at 20 × magnification with 10 scattered fields of view.

⚠ CRITICAL: Because of uneven temperature and CO_2 concentrations among wells in the same plate, try to avoid using the wells on each edge of the CellCarrier-96 plate.

l. Data analysis

Images were analyzed with harmony software from Perkin Elmer. Nuclear areas were defined by DAPI staining, and GFP/cGAS intensity was measured in the defined region of interest. The

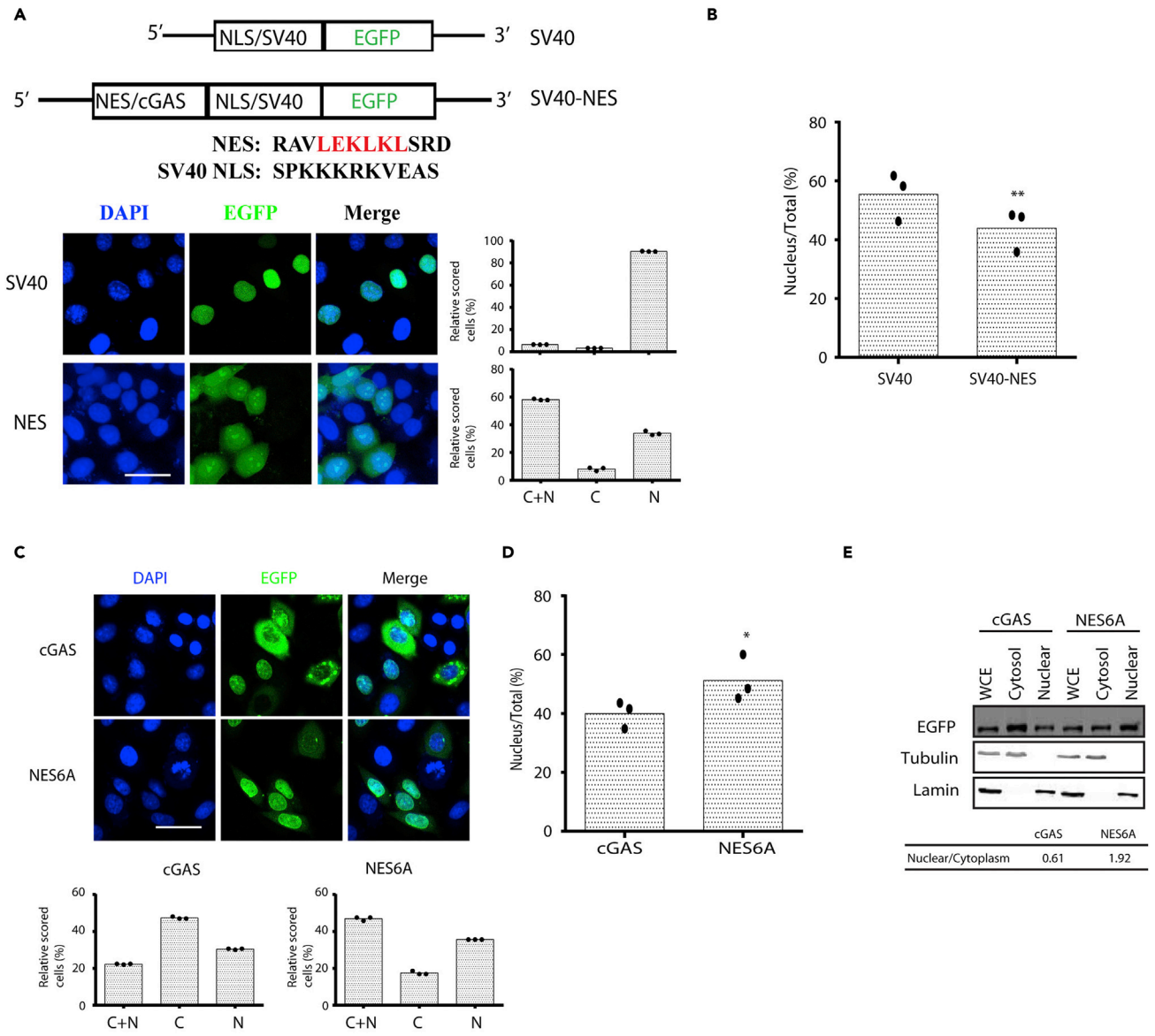

Figure 1. Identification of a functional nuclear export signal in cGAS

(A) cGAS NES directs nuclear export of EGFP. Schematic presentation of the EGFP protein fusion with the NLS from SV40 large T antigen and/or the NES of cGAS. The SV40/SV40NES EGFP signals were observed by confocal microscopy 24 h after transfection. Graphs show mean ± SEM (n=3 independent experiments) representing six different microscopic fields with over 200 cells. Scale bars, 20 μm.

(B) The Operetta High-Content Screen system (PerkinElmer) was used to calculate the ratios of transfected EGFP DNA fluorescence signals between the nucleus and cytoplasm in HeLa cells shown in (A). N, predominantly nuclear; C, predominately cytosolic; C+N, nucleus and the cytoplasm. Relative scored cells are presented as the percentages of N, C or N+C containing cells in all EGFP-positive cells. The results are summarized in the bar graphs (n=3 independent experiments).

(C) Subcellular localization of cGAS mutant NES6A in HeLa cells 24 h after transfection. N, predominantly nuclear; C, predominately cytosolic; C+N, evenly distributed in the nucleus and the cytoplasm. Relative scored cells are presented as the percentages of N, C or N+C containing cells in all EGFP-positive cells. Graphs show mean ± SEM (n=3 independent experiments) representing six different microscopic fields with over 200 cells. Scale bars, 20 μm.

(D) The Operetta High-Content Screen system was used to calculate the ratios of fluorescence signals between the nucleus and cytoplasm for cGAS and its mutant NES6A. The results are summarized in the bar graphs (n=3 independent experiments).

(E) Subcellular localization of cGAS and its mutant NES6A were examined by nuclear and cytoplasmic protein extraction experiment 24 h after transfection. P values of statistical significance are represented as **P <0.01,*P<0.05. These data are from the original Figures 3B–3F in (Sun et al., 2021)

cytoplasmic region of interest (ROI) for GFP/cGAS fluorescence was measured by a dilation from the nuclear boundary, and only thresholded GFP/cGAS was quantitated in the cytoplasmic region. The background fluorescence value was measured in no GFP/cGAS expression cells, and then setup 3 times of background mean fluorescence intensity as positive signal threshold fluorescence value. The nuclear/ (nuclear+cytoplasmic) ratios were calculated (Figures 1B and 1D).

5. Detection of cGAS subcellular localization by Cytoplasmic and nuclear fractionation (Rosner et al., 2013)
   a. Two wells of stimulated cells in a 6-well plate (about 1×10^6cells/well) were needed for fractionation as 1 sample.
   b. 1 μg/well ISD was transfected with PEI(DNA/PEI=1:2)as described above.
   c. After stimulation, cells were washed with 1 mL sterile PBS, and trypsinized with 200 μL/well 2.5% trypsin-EDTA for 5 min at 37°C.
   d. 200 μL/well complete medium was used to neutralize trypsin. Cells were washed twice with 1 mL PBS by centrifugation for 2 min, at 200×g and 4°C. Discard supernatant.
   e. Resuspend the cell pellet with 1 mL cold sterile PBS, transfer one-third of the volume (about 330 μL) to a new 1.5 mL tube, and save this as samples of whole cells.

   ⚠ CRITICAL: Single cell suspension is critical to ensure data quality in the next steps.
   f. Centrifuge the whole cell sample (WCE) at 200×g for 5 min at 4°C. Discard the supernatant. Add 80 μL ice-cold RIPA buffer to lyse the cells for subsequent immunoblotting (C+N).
   g. Pellet the rest two-third cells at 200×g for 5 min at 4°C, discard the supernatant, and keep the tube on ice.
   h. Add 5 pellet volume (about 250 μL) extraction buffer A to resuspend the cell pellet to single-cell solution with 1 mL tips and confirmed by microscopy.

   ⚠ CRITICAL: A sufficient volume (3–10 pellet volume) of extraction buffer A is important for isolation. Too small (less than 3 pellet volume) a volume is insufficient to break cytomembranes. Too much (more than 10 pellet volume) a volume will dilute the cytoplasmic protein concentration.
   i. Keep the lysed cell samples at 25°C for 2 min, then transfer them to ice rapidly for another 10 min incubation.
   j. Add 10% NP40 (about 25 μL) to cell suspension to obtain a final concentration of 1% NP40, and mix gently with 200 μL tips.

   ⚠ CRITICAL: Make sure to gently mix the cells to avoid breaking the nuclear envelope.

   ⚠ CRITICAL: Keep a consistent number of mixing aspirations between samples.
   k. Perform a low-speed centrifugation for 3 min at 500×g at 4°C to separate the cytoplasmic and nuclear components.
   l. Save 30% supernatant carefully to a new 1.5 mL tube as the cytoplasmic component (C), discard the supernatant and keep the pellet on ice.
   m. The pellet contains low-purity nuclei. Wash the pellet with 300 μL extraction buffer A which contains 1% NP40 at 500×g for 3 min at 4°C.
   n. Discard the supernatant, gently resuspend the pellet with another 300 μL extraction buffer A containing 1% NP40.
   o. Repeat step m and n 3 times.
   p. Resuspend the nuclei pellet with 80 μL extraction buffer B and vortex the sample vigorously for 20 s.
   q. Repeatedly snap freeze-and-thaw samples two times in liquid nitrogen and incubate it on ice for 20 min.
   r. Soluble nuclear proteins were separated by high-speed centrifugation at 20000×g at 4°C for 20 min. Discard pellets and collect the supernatant (N).

s. Western blotting (Wang et al., 2020) was carried out to analyze expression of the corresponding protein in the whole cell lysate (WCE) (in RIPA buffer), cytoplasmic (C), and nuclear (N) fractions with 12.5% SDS-PAGE. Load the same amounts of samples, run the gels at 120 V constant voltage for 20 min in running buffer, followed by 160 V for 40 min. Transfer proteins onto NC membrane at 80 V for 120 min in the transfer buffer. Tubulin and Lamin were detected as marker proteins to indicate the cytoplasmic and nuclear fractions, respectively (Figure 1E).

Nuclear export signal (NES) prediction and verification

Timing: 2 h for step 6

Timing: 1 week for step 7

Timing: 1 week for step 8

Timing: 5 days for transformation of the ligated mix into DH5α competent cells, plasmid DNA preparation, and sequencing to confirm the correct clones

6. Prediction of cGAS NES

The human cGAS protein sequence was download from the database UniProtKB: Q8N884 (http://www.uniprot.org/), and then the amino acid sequence was submitted to the LocNES (http://prodata.swmed.edu/LocNES). The software predicted a putative NES located at amino acid positions 169 to 174.

7. Determine the nuclear export function of the putative cGAS NES
   a. Classical NLS (nuclear localization signal) of the SV40 large T antigen sequence (PKKKRKV) was fused to the N-terminus of EGFP to restrain EGFP expression in the nucleus. Then, the putative cGAS NES sequence was inserted to the N-terminus of SV40NLS-EGFP (Figure 1A).
   b. Vector construction details
      i. Digest 1 μg of pEGFP-N1 plasmid with EcoRI and BamH1 for 1.5 h at 37°C.

pEGFP-N1	1 μg
EcoRI-HF (20 U/μL)	1 μL
BamHI-HF (20 U/μL)	1 μL
10 X Cutsmart buffer	2 μL
ddH_2O	X μL
Total	20 μL

      ii. Gel purify digested pEGFP-N1 uisng Axygen Gel Extraction Kit.
      iii. Anneal and phosphorylate each pair of oligos.

SV40	Oligo P1F (100 μL)	1 μL
	Oligo P1R (100 μL)	1 μL
	10× T4 Ligation Buffer	1 μL
	T4 PNK	0.5 μL
	ddH_2O	6.5 μL
	Total	10 μL

SV40-NES	Oligo P2F (100 μL)	1 μL
	Oligo P2R (100 μL)	1 μL
	10× T4 Ligation Buffer	1 μL
	T4 PNK	0.5 μL
	ddH_2O	6.5 μL
	Total	10 μL

The annealing and phosphorylation reaction with the following thermocycler program:

37°C	30 min
95°C	5 min
ramp down to 25°C at 5°C/min	

Dilute annealed and phosphorylated oligos at a 1:200 dilution with ddH_2O.

iv. Ligation reaction

Digested pEGFP-N1 plasmid	1 μL
Diluted oligo	1 μL
10× T4 Ligase Buffer	1 μL
T4 Ligase (40 U/μL)	1 μL
ddH_2O	6 μL
Total	10 μL
Incubate at 16°C for 12 h.	

v. Transformation into DH5α bacteria, plasmid DNA preparation, sequencing to confirm the correct clones.

c. Plasmids were transfected into HeLa cells with PEI. Immunofluorescence microscopy (Figure 1A) and Operetta high-content screen (Figure 1B) were performed 24 h after transfection, to observe and quantify the subcellular distribution of EGFP carrying both NLS and the putative cGAS NES.

8. Function of the putative cGAS NES in mediating cGAS nuclear export

a. Construction of cGAS (NES6A)-EGFP mutant

Putative cGAS NES sequence ^{169}LEKLKL^{174} were replaced by alanines, named cGAS (NES6A)-EGFP.

cGAS (NES6A)-EGFP construction details (overlap)

i. Primer design and synthesis

ii. Upstream and downstream PCR, respectively

Upstream PCR mix	
Template (pEGFP-N1-cGAS plasmid)	1 μL (about 50 pg)
cGASE-F	1 μL
NES6A-R	1 μL
KOD Mix	10 μL
ddH_2O	7 μL
Total	20 μL

Downstream PCR mix	
Template (cGAS-EGFP plasmid)	1 μL (about 50 pg)
NES6A-F	1 μL
cGASE-R	1 μL
KOD Mix	10 μL
ddH2O	7 μL
Total	20 μL

PCR program			
Step	Temperature	Time	Cycle
1	98°C	5min	1 cycle
2	98°C	30s	Step 2–4 30 cycle
3	57°C	30s	
4	68°C	1min	
5	68°C	1min	1 cycle
6	4°C	Hold	
Gel purification of the PCR DNA products using Axygen Gel Extraction Kit.			

iii Overlap extension PCR

Mix 1		
Components		Volume
Template	Upstream DNA	1 μL
	Downstream DNA	1 μL
KOD Mix		10 μL
ddH_2O		6 μL
Total		18 μL

PCR program			
Step	Temperature	Time	Cycle
1	98°C	5min	1 cycle
2	98°C	30s	Step 2–4 10 cycle
3	57°C	30s	
4	68°C	1min	
5	68°C	1min	1 cycle
6	4°C	Hold	

At the end of the program, add primers cGASE-F and cGASE-R to Mix 1 and vortex. Set up the thermocycler for following program:

Step	Temperature	Time	Cycle
1	98°C	5min	1 cycle
2	98°C	30s	Step 2–4 30 cycle
3	57°C	30s	
4	68°C	1min	
5	68°C	1min	1 cycle
6	4°C	Hold	

Purification of the amplified cGAS-NES6A DNA fragment with gel extraction, digestion with EcoRI-HF/BamHI-HF restriction endonucleases

iv. Ligation reaction

Digested pEGFP-N1 plasmid	1 μL
Digested cGAS NES6A DNA	7 μL
10X T4 Ligase Buffer	1 μL
T4 Ligase (40 U/μL)	1 μL
Total	10 μL

Transformation of the ligated mix into DH5α competent cells, plasmid DNA preparation, and sequencing to confirm the correct clones.

b. 1 μg cGAS (NES6A)-EGFP or wild-type cGAS-EGFP DNA was transfected to HeLa cells. After 24 h, cells were fixed with 4% paraformaldehyde (PFA). Confocal imaging (Figure 1C), high content imaging analysis (Figure 1D) and cell fractionation experiments (Figure 1E) were performed to measure the subcellular localization of cGAS and its NES6A mutant.

EXPECTED OUTCOMES

Identify the putative NES in human cGAS using the LocNES Server. Determine the nuclear export activity of this putative NES using either a reporter protein (EGFP) or on endogenous cGAS, through construction of the NES-SV40-EGFP and cGAS-NES6A mutants. Detect and quantify the subcellular localization of cGAS and corresponding NES mutants with immunofluorescence microscopy, Operetta high-content screen, and cytoplasmic/nuclear fractionation. The results are expected to demonstrate that the NES of cGAS is able to direct the nuclear export of SV40NLS-EGFP and that nuclear localization of cGAS is lost when this NES is mutated.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments of IF, Operetta high-content screen and cytoplasmic/nuclear fractionation were performed three times independently. Data were plotted as mean values, with variation as s.e.m. Student's two-tailed t test was used for Statistical significance calculated.

LIMITATIONS

LocNES (http://prodata.swmed.edu/LocNES) is a database used for classical leucine-rich nuclear export signal prediction, which is mediated by chromosomal region maintenance 1 (CRM1). However, non-classical CRM1-mediated NESs have been reported in recent years, and is not covered by this database.

GFP-NLS system may be artificial and fusion position and distance of NES to this may affect results. The fusion position and distance maybe affected function of NES and other manners of fusion could be tried to verify the effect of cGAS NES.

cGAS cellular location was dependent on several factor such as viral infection, cell cycle, DNA damage. Nuclear located cGAS is recruited to double-stranded breaks (DSBs), suppresses DNA repair, and post-translational modification such as Y215 phosphorylation is critical for cGAS cytosolic/nuclear translocation.(Liu et al., 2018).Whether Y215 phosphorylation of human cGAS was affected by cGAS NES mutation should be verification.

Operetta high-content screen and cytoplasmic and nuclear fractionation only measure the average change of signals. More accurately measuring the signal of cGAS subcellular location in single cells is not easily achievable. It was noted that cGAS can be present in both the cytoplasm and the nucleus and that the relative abundance of these two cGAS pools can vary between cells. This can add variation when quantifying the subcellular distribution of cGAS.

As a DNA sensor, cGAS is a shuttle protein transported between nucleus and cytoplasm. The transfected vector DNA may induce cGAS cytoplasmic localization and lead to cellular distribution variation. Additionally, subcellular location of cGAS is affected by cell cycle, and tethered by chromatin during mitosis (Li et al., 2021). Therefore, cell state is important for its cellular localization.

TROUBLESHOOTING

Problem 1

Cell death in transfection or stimulation in step 1 and step 2.

Potential solution

Reduce the amount of ISD in transfection or reduce the amount of transfection reagent used in preparation of transfection mix. Reduce the amount of inactivated VACV amount. Or shorten the stimulation time.

Problem 2

The fluorescence signal is not clear in step 3 of immunofluorescence microscopy and Step 4 Operetta high-content screen.

Potential solution

a. cGAS knockout cell lines can be used to validate antibody specificity.

b. This can also be caused by overlapped cells. Density of cells is important for fluorescence signal acquisition. We recommend a relatively low cell density to ensure monolayer cell culture and sharpness of cell boundary. In the meantime, too few cells may cause large variations in quantification.

Problem 3

Large variation in step 4 of high-content data

Potential solution

Optimize the transfection protocol. Reduce the error of pipetting by increasing reaction volume. Digest cells sufficiently and gently shake the 96-well plate to make sure cells distribute evenly.

Problem 4

Incomplete separation in step 5 of nucleus and cytoplasm

Potential solution

Cytoplasm components may contaminate the nuclear fraction, which can partly be a result of insufficient wash of the nuclear pellets. The amount of extraction buffer used is crucial. Increasing the volume of wash buffer and the wash times can improve the purity of the samples. However, excessive washing can lead to the loss of the nuclear samples.

Problem 5

No obvious cGAS band in step 5 of nucleus and cytoplasm fractionation

Potential solution

Increase the amount of sample cell. Increase the primary antibody concentration and incubation time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fei Guo (guofei@ipb.pumc.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate and/or analyze any datasets.

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AUTHOR CONTRIBUTIONS

Y.H. and M.M. composed the manuscript. C.L. and F.G. discussed, reviewed, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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