**Figure 3-1. Establishment of biochemical assay for purification of cGAS activity regulator(s).**

(A) Experimental design of the in vitro assay to test the activity of cGAS and its putative inhibitor(s)

(B) Representative data from thin layer chromatography analysis showing the synthesis and detection of cGAMP. Organic running buffer (left) or inorganic running buffer (right) was used to separate inorganic phosphate and cGAMP.

(C) Representative data from PFO permeabilization mediated cGAMP delivery assay. Cytosol (S20) from L929 cells was used as cGAS activity provider in assay shown in (A), the reaction mix was boiled and delivered to THP-1 reporter cells using PFO. cGAMP standard was also delivered as controls.

(D) L929 and THP-1 cell can produce cGAMP in response to DNA transfection. L929 and THP-1 cells are transfected with 2ug/ml HT-DNA for 3 hours. Cells are collected and lysed in hypotonic buffer. Cytosol was heat inactivated by boiling at 95 degrees for 5 minutes and the supernatant was delivered to THP-1 reporter cells with PFO permeabilization.

(E) THP-1 cytosol (S20) shows no detectable cGAS activity. L929 and THP-1 cytosol was collected using hypotonic buffer lysis and subjected to cGAS activity test as shown in (A). The reaction mix was boiled and delivered to THP-1 reporter cells.

(F-G) THP-1 cytosol (s20) shows cGAS inhibitory activity. L929 cytosol (F) or 3ng human recombinant cGAS (G) showed cGAS activity (No inhibitor groups). Adding THP-1 s20 into the reaction can inhibit the activity of cGAS in dosage dependent manner.

**Figure 3-2. Characterization and purification of a putative cGAS inhibitor in THP-1 cell lysate.**

(A) The putative cGAS inhibitor from THP-1 cytosol is further induced by ligand stimulations. THP-1 cell were treated with HT-DNA (2ug/ml), poly(I:C) (2ug/ml), HSV (MOI=5), or left untreated before cytosol (s20) was collected. The concentration of cytosols was adjusted to 6ug/ml and indicated volume of cytosol was used for cGAS inhibitory activity test.

(B) Putative cGAS inhibitor(s) from THP-1 cytosol is heat-sensitive. Inhibitory THP-1 cytosol was heated at 95 degrees for 5 minutes and its cGAS inhibitory activity was tested.

(C) Effect of adding more DNA, ATP, or GTP to the cGAS reaction. In control reaction (1x All), 10ng/ul HT-DNA, 1mM ATP, and 0.5mM GTP were used. In 10x DNA reaction, 100ng/ul HT-DNA was added; in 10x ATP reaction, 10mM ATP was used; and in 10x GTP reaction, 5mM GTP was added to reaction.

(D) Purification methods and their purification folds tested is summarized in the table. Those with good purification effects were labeled as red.

(E) RNase can greatly reduce the inhibitory activity in THP-1 cytosol. THP-1 cytosol was left untreated or treated with RNase A and RNase T1 cocktail on ice for 1 hour. Then indicated amount of treated and untreated cytosol were tested in cGAS activity assay.

(F) Inhibitory fraction eluted from Hitrap Q column is sensitive to RNase activity. THP-1 cytosol was loaded on Hitrap Q column and eluted with gradient concentration of NaCl. The fractions were collected and tested for cGAS inhibitory activity. The inhibitory fraction was left untreated or treated with RNase cocktail, RNase A, or Cyanase, after which the indicated amount of fraction was tested in cGAS activity assay.

**Figure 3-3. Certain RNA species can inhibit cGAS activity in vitro.**

(A) RNase can greatly enhance the production of cGAMP in cGAS activity assay. Increasing amount of human recombinant cGAS, L929 cytosol (s20), or THP-1 cytosol was tested in aGAS activity assay in presence of absence of RNase cocktail.

(B-C) Total RNA extracted from THP-1 cell and 293T cell can inhibit cGAS activity in vitro. Total RNA was isolated from THP-1 cell (Ctl), Sendai virus treated THP-1 cell (SeV), 293T cell, or inhibitory fraction eluted from Hitrap Q column (Q\_100%B) and tested in cGAS activity assay. The cGAS inhibitory effect was evaluated by THP-1 reporter cell assay (B) or TLC assay (C).

(D) RNA from different source can inhibit cGAS activity. RNA extracted from human recombinant sumo-cGAS, E.Coli ribosomes, rabbit reticulocytes, or inhibitory fraction eluted from Hitrap Q column (Q\_100%B) was tested in cGAS activity assay in presence or absence of RNase.

(E-F) 23S and 16S ribosomal RNA from E.coli can potently inhibit cGAS activity. E.coli ribosomal RNA was separated on agarose gel (F) and bands representing different subunits were cut and extracted. The indicated amount of RNA was added to test in cGAS activity assay.

**Figure 3-4. Short synthesized RNA oligos inhibit cGAS activity in sequence and length dependent manner.**

(A) Synthetized dsRNA R reserve strand but not forward or annealed double stranded RNA can inhibit cGAS activity in vitro. Forward (F) or reverse (R) RNA was synthesized with sequences shown at bottom. Indicated amount of RNAs was tested in cGAS activity assay.

(B-C) Kinetics for cGAS inhibition by RNA oligo. Indicated amount of RNA oligos and ISD were added in the cGAS activation assay. The result (B) was quantified with ImageJ software and showed in (C)

(D-E) Inhibition of cGAS by RNA oligo is dependent on its length. Indicated truncated RNA oligos were added in the cGAS activity assay.

(F) Single stranded DNA with the same sequence show no inhibitory effect on cGAS activity. DNA oligos with indicated sequences were added in the cGAS activity assay.

(G) Summary of sequences of RNA oligos test in cGAS activity assay. Those with potent inhibitory effect were labelled as red.

(H) Alignment of regions from HIV polymerase and SIV polymerase coding sequence.

(I) In vitro transcribed HIV genome RNA can potently inhibit cGAS activity. The indicated fragments from HIV genome was transcribed in vitro. Indicated amount of the RNA was added to evaluate their cGAS inhibitory effect.

(J) Caspase 9 mRNA can potently inhibit cGAS activity. Commercially purchased mRNA encoding Caspase 9 was added to cGAS activity assay in presence of absence of RNase.

**Figure 3-5. Phosphorothioate bond DNA oligos inhibit cGAS activity.**

(A) DNA oligo with phosphorothioate bond but not phosphodiester bond backbone can inhibit cGAS activity in vitro. The oligos with the indicated sequences was added evaluate their cGAS inhibitory activity.

(B-C) Kinetics for cGAS inhibition by Phosphorothioate bond DNA oligo. A titration of indicated oligos were added in cGAS activity assay. The result (B) was quantified with ImageJ software and showed in (C).

(D) Phosphorothioate bond DNA oligo can inhibit cGAS activation by ISD transfection. ISD and CpG DNA oligos were transfected to bone marrow derived dendritic cells individually or in combination as indicated, for 4 hours. RNA was isolated. IFN-b, IFN-a4, and IL-6 mRNA levels were analyzed by q-RT-PCR.

**Figure 3-6. The majority of cGAS in dividing cells presence in the heavy fractions and is resistant to detergent extraction.**

(A) The majority of cGAS is resistant to detergent extraction in THP-1 cells. THP-1 cell (THP-1 wt) and THP-1 cell with cGAS knocked out and rescued with Flag tagged cGAS (THP-1 cGASKO\_FG-cGAS) were collected and suspended in PBS or PBS supplemented with PFO or NP-40. After incubation, the cells were centrifuged at 20,000g for 5 minutes and the supernatant and pellet were collected for Western Blot analysis.

(B) High salt can extract cGAS from THP-1 cells. THP-1 cells were first lysed in hypotonic buffer and cytosol protein (s20) was collected. For the pellet was further extract with RIPA buffer (RIPA\_ext). The pellet left from RIPA buffer extraction was extracted with hypotonic buffer supplemented with 1M NaCl (hi-salt extract). The pellet from the 1M NaCl extraction was boiled in SDS loading buffer. All fractions were processed for Western Blot analysis.

(C) High salt can extract cGAS from Hela cells. Hela cells (Hela\_wt) and Hela cGAS knockout cells (Hela\_cGAS KO) were subjected to the same sequential extraction in (B) and different fractions were analyzed with Western Blot for cGAS distribution.

**Figure 3-7. cGAS is recruited to chromosomes during mitosis and is present in the nucleus in actively dividing cells.**

(A) Strong GFP-cGAS signal detected in Hela\_GFP-cGAS cell line. Hela cell stably expressing GFP (Hela\_GFP) or GFP tagged cGAS (Hela\_GFP-cGAS) were visualized with florescence microscope with DAPI nuclear staining.

(B) GFP signal perfectly co-localize with nuclear content in Nocodazole synchronized cells. Hela\_GFP and Hela\_GFP-cGAS cells were first synchronized with Nocodazole for 12 hours before visualized with florescence microscope with DAPI nuclear staining.

(C) Co-localization of GFP-cGAS with chromosome/chromatin at different stages of division. Hela\_GFP and Hela\_GFP-cGAS cells were synchronized at G1/S phase with double thymidine protocol and then release for mitosis progress. Cells at different stages were visualized with confocal microscope with DAPI nuclear staining.

**Figure 3-8. Chromatin high salt extract contains cGAS inhibitor.**

(A) cGAS from both cytosol and nucleus can be activated in DNA dependent manner. Hela cell stably expressing Flag tagged cGAS (Hela\_FG-cGAS) was lysed in hypotonic buffer. The cytosol and nucleus fractions were collected and tested for cGAS activity in presence or absence of ISD.

(B) High salt extraction from nucleus can inhibit cGAS in vitro. Hela cell stably expressing Flag tagged cGAS (Hela\_FG-cGAS) was lysed in hypotonic buffer. Then hypotonic buffer supplemented with 0.5M NaCl was used to extract the nucleus (0.5M NaCl extract). The cGAS inhibitory effect of the 0.5M NaCl extract was evaluated (as control, hypotonic buffer with 0.5M NaCl was used)

(C-D) Putative cGAS inhibitor from nucleus high salt extract co-immunoprecipitate with cGAS. Hela\_FG-cGAS cells was lysed in hypotonic buffer and cytosol protein was collected (s20), the nucleus was further extracted with hypotonic buffer supplemented with 1M NaCl (1M NaCl extract); both s20 and 1M NaCl extract were used for subsequent imnunoprecipitation with M2 agarose beads in buffer with 0.4M NaCl or 1.0M NaCl respectively. Flag peptide containing buffer was used to elute FG-cGAS and its binding protein and the eluates, along with the starting materials were evaluated for cGAS activity. The amount of cGAS in starting materials and in IP eluates was evaluated by Western Blot (D).

(E) Putative cGAS inhibitor from nucleus cGAS IP can be eluted by buffer with 1M NaCl. Similar immunoprecipitation experiment as in (C) was performed, except that the IP was only performed in 1M NaCl buffer and that the beads were eluted with hypotonic buffer supplemented with 1M NaCl and BSA carrier protein.

(F) Putative cGAS inhibitor is sensitive to protease, but not RNase or DNase treatment. The IP eluates from E was either boiled at 95 degree for 5 minutes or treated with DNase, RNase, or Protease K at 37 degree for one hour. The cGAS inhibitory activity was evaluated.