CHAPTER THREE

Characterization and Purification of cGAS inhibitor(s)

Certain RNA as cGAS inhibitor

Results

Establishment of biochemical assay for purification of cGAS activity regulator(s).

To purify and identify regulators for cGAS activity, we modified a previously established a cell-free cGAMP synthesis assay. Briefly, as shown in Figure 3-1A, when we mix cGAS activity provider, which can be either recombinant cGAS protein or L929 cell lysate (s20, supernatant of 20,000g centrifugation), with DNA, ATP and GTP, the reaction would synthesize cGAMP when incubated at 37 degrees. Then the synthesized cGAMP can be delivered into THP1-Lucia™ ISG cells (a cell line that expresses the secreted luciferase (Lucia) reporter gene under the control of an IRF-inducible promoter, allowing the monitoring of the IRF pathway by determining the activity of Lucia luciferase in the cell culture supernatant) at 1:10 dilution in the culture medium with PFO permeabilization (25ng/ml perfringolysin O), and the concentration of cGAMP in the reaction was evaluated by comparing the Lucia signal with cGAMP standards that were delivered into the reporter cells. Figure 3-1C shows an example of the readout for this assay. We can see that with 40ug of L929 s20, it can produce about 30nM of cGAMP in the 40ul reaction mixture. Alternatively, we incorporate [α-32P]-ATP in the reaction and after reaction ends, the 32P labeled product can be separated and analyzed by thin layer chromatography (TLC) assay. Figure 3-1B shows two example TLC runs. Depending on the kinds of TLC plates and running buffer, cGAMP could run either above (left panel) or below (right panel) the inorganic phosphate. With the assays described above, we can add cell lysate or its fractions into the reaction and test its regulation to cGAS activity by measuring the concentration of cGAMP after reaction being terminated by 95 degrees heating for 5 minutes.

In cells with intact cGAS-STING DNA sensing pathway, when transfected with interferon stimulatory DNA, IRF3 dimerization and type I interferon production can be detected (data not shown). To show that cGAS is activated by DNA transfection, we transfected L929 and THP-1 cells with 2ug/mL ISD and 2 hours post transfection, the cells were collected and lysed in hypotonic buffer. The cell lysate was heated at 95 degrees for 5 minutes and was delivered into the THP-1 reporter cells to measure the cGAMP concentration. In Figure 3-1D, we can see that in both cell lines, cGAMP was produced with DNA transfection. Interestingly, however, when we isolated the cytosol (s20) from L929 and THP-1 cells and applied them to the in vitro cGAS activity assay described above, we can only detect cGAS activity in L929 lysate (Figure 3-1E), even though there was relatively high level of cGAS protein in THP-1 cell lysate (data not shown).

To check if there were potential regulatory factor(s) in THP-1 lysate, we investigated the regulatory effect of the lysate on cGAS activity. We found that with increasing amount of THP-2 cytosol, the cGAS activity from both L929 s20 (Figure 3-1F) and from human recombinant cGAS (Figure 3-1G) were gradually inhibited. This suggests that certain components in THP-1 cell lysate can inhibit the activity of cGAS in in vitro reactions, which also explains why we were not able to detect cGAS activity in THP-1 lysate.

Characterization and purification of a putative cGAS inhibitor in THP-1 cell lysate.

To test if this cGAS inhibitory activity in THP-1 was regulated in cells, we first treated THP-1cells with HT-DNA, and found more potent cGAS inhibitory effect in the cell lysate (Figure 3-2A). To investigate if this induction effect was specific to DNA stimulation, we also treated THP-1 cell with poly(I:C) or HSV before extract cytosol for inhibitory effect tests. The result shows that all these treatments can enhance the cGAS inhibitory activity in THP-1 cytosol, suggesting this factor could be interferon inducible. This might be a positive feedback regulation mechanism that cells utilize to shut down the immune response after the downstream signal has been properly activated.

To characterize the inhibitory factor, we did several tests to show that the inhibitor activity in the THP-1 lysate was not due to protease(s) that degrade cGAS protein (data not shown), that the factor was sensitive to heat (95 degrees for 5 minutes, Figure 3-2B), and that it did not inhibit cGAS activity through competing against cGAS-DNA binding (Figure 3-2C). Interestingly, we found that adding more than 1mM ATP alone in the reaction inhibited cGAS activity.

We then tried to fractionize THP-1 cell lysate using different methods or with FPLC columns. Table in Figure 3-2D listed the methods and columns and labeled the ones with good purification efficiency red. The fact that the factor bound to Q and CHT columns suggested that it was negatively charged and that it was rich in phosphate group. However, we also found that the inhibitor we were looking for formed huge complex and was eluted at the void volume on SuperoseTM 6 columns (suggesting a molecular weight of heavier than 5 x 106 Dalton). And as we tested, the cGAS inhibitory complex was resistant to high salt (up to 2M NaCl), detergent (up to 1% sodium deoxycholate and 0.5% SDS) (data not shown). Surprisingly, we found that either nucleases, like Cyanase, or RNase could break the large complex (as shown on gel filtration columns, data not shown). And we can see that RNase or Cyanase treatment could kill the cGAS inhibitory effect in THP-1 s20 (Figure 3-2E) and also in its inhibitory fraction eluted from Hitrap Q column (Figure 3-2F). These results above strongly indicated that the inhibitor we were looking for should be certain RNA species.

Certain RNA species can inhibit cGAS activity in vitro.

To confirm that RNA can inhibit cGAS activity in vitro, we treated THP-1 s20 with RNase, expecting to release the cGAS in the lysate from inhibition. However, the RNase treatment could not restore cGAS activity in the cytosol (Figure 3-3A), probably because RNase could not degrade all the inhibitory RNA in the lysate (since cGAS activity is very sensitive to temperature, we could only treat the sample with RNase at 4 degrees). Yet, we did find that with RNase included in the reaction mix, the activity of human recombinant cGAS or cGAS from L929 s20 increased dramatically. This was due to that the residual RNA in the purified cGAS and in the L929 lysate can inhibit the cGAS activity in some extent.

We also purified total RNA form THP-1 cells (either control cells or cells treated with Sendai virus), 293T cells or from inhibitory fraction eluted from Hitrap Q column and found that total RNA from all these cells could inhibit cGAS activity in vitro (Figure 3-3B and C). Also, we tested RNA extracted from E. coli ribosome, rabbit reticulocytes, or from purified recombinant cGAS from E. coli, all of which could inhibit cGAS activity and including RNase in the reaction fully released the inhibition (Figure 3-3D). Further, we separated E. coli ribosome RNA on agarose gel (Figure 3-3F), cut the bands for different subunits, extracted/purified the RNA and found that both 23S and 16S showed potent inhibition to cGAS activity. The inhibition effect of 5S subunit was relatively weak.

Short synthesized RNA oligos inhibit cGAS activity in sequence and length dependent manner.

To further test the inhibition of cGAS activity by RNA, we synthesized and tested series of 45 nucleotide single stranded RNA (ssRNA). Interestingly, one of the RNA sequences, which was consist of random A and C combination (ssRNA F), showed no inhibition effect while its complimentary reverse sequence (ssRNA F) showed very potent inhibition to cGAS activity in vitro (Figure 3-4A). Notably, the double stranded RNA (dsRNA) annealed from the ssRNA above did not inhibit cGAS activity. In Figure 3-4B, we tested the kinetics for cGAS inhibition by RNA. We titrated the amount of both ssRNA and ISD used in the reaction and used ssRNA F as negative control. The data was analyzed and quantified by ImageJ software (Figure 3-4C). With 24ng (20nM) cGAS and 100ng (166nM) ISD in the reaction, the IC50 for the inhibitory RNA oligo was 10.41ng (30nM). And when ISD concentration increased by 10-fold, the IC50 only doubled to 20.60ng (68.37nM).

To test if the inhibition of cGAS activity by RNA dependent on the length of cGAS, we truncated the original 45nt ssRNA R (also labeled as RNA\_001) and tested if the RNA oligo of shorter version could inhibit cGAS activity. Surprisingly, deleting only 5 nucleotide from the original sequence largely killed the inhibitor activity (Figure 3-4E, RNA\_004) and further truncation would totally abolish the inhibitory effect (Figure 3-4D). We also synthesized the DNA oligos with the same sequence as the inhibitory RNA and as expected, the single stranded DNA oligo did not inhibit cGAS activity (Figure 3-4F).

The inhibition of cGAS by RNA seemed to be dependent on the sequence of the RNA. In order to test this, we synthesized series of RNA oligos, either found in the database that share sequence similarity to RNA\_001 or with sequence derived from RNA\_001 with small changes. The sequences of oligos tested are listed in Figure 3-4G and the ones with potent inhibitory effect are labeled in red. Of the listed sequence, one striking data was that comparing RNA\_009 and RNA\_012, which were selected sequence from polymerase coding sequence of HIV and SIV, respectively. From the alignment in Figure 3-4H, we could see that the two RNA share very similar sequences, however, as RNA\_009 had very strong cGAS inhibition activity while RNA\_012 could not inhibit cGAS activity at all.

With the data above, we were interested to see if specific RNA region from virus origin can inhibit cGAS and regulate host immune response. We in vitro transcribed HIV genome RNA fragments (2 kilobases for each fragment). Surprisingly, all fragments can potently inhibit cGAS activity in vitro (Figure 3-4I). We further tested some random long mRNA sequences (Cas9 mRNA, as an example in Figure 3-4J) and found that they all had cGAS inhibition activity. This suggests that when reaching certain length, single stranded RNA can inhibit cGAS in sequence independent manner.

Phosphorothioate bond DNA oligos inhibit cGAS activity.

Previous research reported that Synthetic oligodeoxynucleotides with phosphorothioate (PS) bond containing suppressive TTAGGG motifs could abolish DNA sensing response by inhibiting AIM2 inflammasome activation (Kaminski et al. 2013). So, we were interested in finding out if phosphorothioate bond DNA oligos would inhibit the activity of cGAS. We synthesized the PS bond and phosphodiester (PO) bond DNA oligos, and also RNA with TTAGG repeats (A151) and control sequence (C151), as described in the publication above. Interestingly, we found that both P-S bond DNA can inhibit cGAS activity but not PO bond DNA or RNA (Figure 3-5A). We did a titration of the PS bond DNA with sequence of random A’s and C’s and found it had an IC50 of 135nM when cGAS was 30nM in the reaction (Figure 3-5B and C).

To further test if the PS bond DNA oligo could inhibit cGAS in cells, we co-transfected ISD with CpG DNA oligos and checked the induced expression of type-I interferons and inflammatory cytokines (Figure 3-5D). As expected, ISD alone transfection in conventional dendritic cells induced high mRNA levels of IFN-b, IFN-a4, and IL-6, which dependent on the DNA sensor, cGAS. Surprisingly, adding CpG DNA oligos (either CpG-B or CpG-C) would totally abolish the induction. These results suggest that PS bond DNA can inhibit cGAS activity both in vitro and in cells.

Conclusions and discussion

In this part, we discovered that certain RNA species in from selected cell lines can inhibit the activity of cGAS in vitro. This putative cGAS inhibitory RNA can be further induced by interferon, which may indicate that a regulatory feedback loop could exist in cells to control the activity of cGAS after being activated.

It’s surprising that single stranded RNA could potently inhibit cGAS. As shown in Figure 3-3E, for E.coli 16S and 23S ribosomal RNA, as little as 10ng (0.9nM and 1.7nM respectively for 23S and 16S) could almost totally abolish the activity from reaction with 30nM cGAS with 220nM ISD in the mixture. This inhibition could due to that ssRNA can bind with cGAS. Indeed, with electrophoretic mobility shift assay (data not shown), we found that ssRNA can bind with purified cGAS, with similar or a little bit higher affinity than double stranded DNA. However, all RNA oligo observed, including inhibitory, non-inhibitory ssRNA, and dsRNA, can bind to cGAS. Probably because RNAs are negatively charged while cGAS is positively charged at physiological pH. The inhibition of cGAS by RNA dependent on the length of the oligo and the inhibition very sequence sensitive when the oligo is short. But when the RNA is long, it can inhibit cGAS activity without sequence specificity. This raises the question that why in cells, with all the ribosomal RNA and mRNAs presence in cytosol, how cGAS can be activated by DNA. A possible explanation could be that for the majority of ribosomal and mRNAs, they are bound by RNA-binding proteins and thus kept away from cGAS.

We have not been able to purify or identify the exact RNA species that act as the physiological cGAS inhibitor in cells. There have been the following difficulties. The first is that there are no effective methods to fractionate and purify specific RNAs. We did try to pull down cGAS binding RNAs through co-immunoprecipitation with or without cross-linking. However, there were always contaminations from ribosomal and mRNAs, which were difficult to get rid of. And we also tried to understand the mechanism by which RNA inhibit cGAS by cGAS-RNA co-crystallization. However, we were not able to get crystals with good quality, probably due to that the inhibitory RNAs were too long for crystallization and we could not truncate the RNA while preserving its inhibitory activity.

cGAS inhibitor(s) from nucleus

Results

cGAS is considered to be the cytosolic DNA sensor due to the fact that it was originally purified and identified in the cytoplasm. This raised the question that during cell cycle, when nucleus membrane breaks down, why cGAS is not activated if it can bind chromosome DNA. In the beginning, a possible explanation was that during M phase, genomic DNA is highly condensed to chromosomes and cGAS would not be able to access to chromosomal DNA. However, here we discovered that after nuclear membrane breaks down, cGAS is recruited to chromosome and resides in the nucleus after mitosis finishes and nuclear membrane re-established. This leads to a surprising phenotype that in cultured cell with active cell division, the majority of cGAS is localized in the nucleus. So, it is interesting to investigate how the activity of cGAS is regulated in the nucleus and whether nuclear cGAS has any physiological functions.

The majority of cGAS in dividing cells presence in the heavy fractions and is resistant to detergent extraction.

In experiments that we separated different fractions of cell lysate using differential centrifugation, we unexpected found that instead of being in the supernatant of 20,000g centrifugation, as most cytosol proteins do, cGAS was located at very heavy fractions (data not shown). Further we tested different methods to permeabilize or break the cells to extract cGAS, as shown in Figure 3-6A. We did the test in both THP-1 wild type cells and also THP-1 cGAS knockout cells that were rescued with Flag tagged cGAS. With collected cell pellet, we used PBS, or PBS with PFO (which only permeabilize cell membrane and thus only extract cytosolic proteins to supernatant) or with NP-40 (which should extract both cytosol proteins but also proteins from membrane organelles). As expected, ikb-alpha, which is a cytosolic protein, can be isolated by both PFO and NP-40 containing PBS. MAVS, as a mitochondria protein, can only be efficiently extracted by NP-40 containing buffer. However, to our surprise, cGAS, which we considered to be a cytosolic protein, was resistant to both PFO and NP-40 extraction. Further, we used sequential extraction methods to isolate proteins from different cellular components (Figure 3-6B). We first used hypotonic buffer to break the cell membrane and extracted cytosols (s20), then with the pellet (p20) from hypotonic buffer extraction, we used Radioimmunoprecipitation assay buffer (RIPA buffer) to extract both membrane organelle proteins and nuclear proteins. Finally, we used 1M NaCl buffer to extract proteins that were in the pellets from RIPA buffer extraction. As shown in Figure 3-6B, ikb-alpha and MAVS proteins are in s20 and RIPA buffer extract respectively. While there was a small amount of cGAS protein in the cytoplasm, the majority of cGAS could only be extracted with 1M NaCl (hi-salt extract). Similarly, we also tested this on Hela cGAS knockout cells and Hela wild type cells and cGAS showed similar pattern with extraction (Figure 3-6C). These results above suggest that cGAS is not a “simple” cytosolic protein. It could either be forms heavy, detergent resistant complex in the cytosol, or it could be localized in the nucleus.

cGAS is recruited to chromosomes during mitosis and is present in the nucleus in actively dividing cells.

To further study the localization of cGAS in cells, we stably expressed GFP tagged cGAS in Hela cells (Hela\_GFP-cGAS cell line, and as control, we also express GFP protein in Hela cells, Hela\_GFP cell line). To our surprise, in Hela\_GFP-cGAS cells, the nucleus showed very strong GFP-cGAS signal, indicating that indeed most of the protein was localized in the nucleus (Figure 3-7A, left panels). More interestingly, as outlined by rectangular boxes, in dividing cells at anaphase, the cGAS-GFP signal perfectly co-localized with the chromosomes. As control, in Hela\_GFP cells, the GFP signal just showed ubiquitous distributions in both cytoplasm and nucleus (Figure 3-7A, right panels).

To confirm that cGAS-GFP would co-localize with chromosomes at metaphase, we treated both cell lines with Nocodazole to synchronize the cells at M phase. After Nocodazole treatment for 12-16 hours, the cells that were synchronized at M phase become rounded and lose most of their attachment to the substratum. And due to lack of polymerized tubulin to form spindles, instead of aligning at the mitotic plate of the cells, the chromosomes form condensed structure that gave very strong signal with DAPI staining (Figure 3-7B). And strikingly, the cGAS-GFP signal in these M phase cells also formed the same condensed structures and exactly co-localized with the DAPI signal. And interestingly, in the cells that were not in M phase after Nocodazole treatment, the majority of cGAS-GFP signal came from cytoplasm. The reason for this observation could be that these cells somehow escaped the Nocodazole induced cell-cycle arrest and were then arrested at G1 phase, during which the localization of cGAS should be mainly in the cytoplasm (as will also be shown below). In contrast, the GFP signal in Hela\_GFP cell line were non-specifically distributed in the whole cells.

To fully understand how the localization of cGAS changes dynamically within a cell cycle, we used double-thymidine method to synchronize the cells at G1 phase, released the arrest so that the cell enters next cycle and monitored the localization of GFP-cGAS at different stages of division (Figure 3-7C). After thymidine treatment for extensively long time (about 36 hours), we can see that the majority of GFP-cGAS was located in the cytoplasm (G1/S phase). About 10 hours after releasing, most of the cells enters M phase. During prometaphase and metaphase, almost all of the GFP-cGAS protein were recruited to and co-localized with chromosomes. In contrast, in Hela\_GFP cells, during prometaphase and metaphase, GFP protein was excluded from chromosomes. When cells entered telophase (nuclear membrane reformed) and cytokinesis (nucleoli reappeared, and chromosomes unwound into chromatin), the majority of the GFP-cGAS protein still presented in the nucleus. As tested, after cytokinesis and cell division completed, if the cell cycle was not arrested, then most of the GFP-cGAS protein was still localized in the nucleus when the next cell cycle began thus leading to the phenomenon that most of the cGAS protein was detected in the nucleus. However, if we arrested the cell cycle in G1/S phase, after 24-36 hours, more and more GFP-cGAS signal appeared in the cytoplasm. This is probably due to the fact that newly translated cGAS protein could not enter the nucleus until nuclear membrane breaks down. Meanwhile the GFP-cGAS signal in the nucleus decreased which could be caused either by the exportation of nuclear cGAS or by the degradation of the cGAS in the nucleus. Overall, these data show that the cellular localization of cGAS is dynamically regulated by cell cycle and in actively dividing cells, most of the cGAS protein actually exists inside the nucleus.

Chromatin high salt extract contains cGAS inhibitor.

Since we detected that the majority of the cGAS in dividing cells is localized in the nucleus, it is intriguing to check if the nuclear cGAS can be activated by chromatin DNA and DNA from other sources. From Hela cells that stably express Flag tagged cGAS (Hela\_FG-cGAS), we used hypotonic buffer to isolate both cytosol and nucleus. Then with or without ISD in the reaction mix, we checked the activity of cGAS in these fractions. As expected, the cytosolic cGAS could be activated in ISD dependent manner (Figure 3-8A, Cytosol). Interestingly, we found that the nucleus cGAS could also be activated by ISD, but it was not activated by the chromatin already in the nucleus (Figure 3-8A, Nucleus). Furthermore, we found that though containing the majority of cGAS in the cells, we were not able to detect any cGAS activity from high salt extract from the nucleus (data not shown). Instead, the high salt extract could further inhibit the activity of recombinant cGAS (Figure 3-8B, 0.5M NaCl extract). This observation indicated that there should be certain cGAS inhibitor(s) in the 0.5M NaCl extract from the nucleus.

To further test if the putative inhibitor in the high-salt extract binds with cGAS, we isolated cell cytosol (s20) and nuclear high-salt extract (1M NaCl extract) from Hela\_FG-cGAS cells. In consistent with previous results, s20 showed good cGAS activity (Figure 3-8C, s20) while the high-salt extract did not exhibit any cGAS activity (Figure 3-8C, 1M NaCl extract). For both s20 and 1M NaCl extract, we performed immunoprecipitation using agarose beads conjugated with M2 antibody at two conditions: one with 0.4M NaCl and the other with 1M NaCl in the immunoprecipitation buffer. After immunoprecipitation, we used flag peptide to elute the binding proteins from the beads. As shown in Figure 3-8C, eluates of s20 immunoprecipitation at both salt conditions showed good cGAS activity. Interestingly, though eluates of 1M NaCl extract immunoprecipitation at both salt conditions resulted in similar amount of cGAS protein (Figure 3-8D), the eluate of immunoprecipitation with 1M NaCl in the buffer showed good cGAS activity while that with 0.4M NaCl showed none. These data suggest that in the high salt extract from the nucleus, there are both cGAS and inhibitor(s) that can suppress cGAS activity. When performing the immunoprecipitation with 1M NaCl, the putative inhibitor was disassociated from cGAS and thus remained in the flow through, and that’s the reason we could detect cGAS activity. With lower ion strength (0.4M NaCl), the putative inhibitor still bound with cGAS and was co-immunoprecipitated. In this case, the inhibitor would also be eluted from the beads along with cGAS.

To further confirm the theory above, we isolated cytosol (s20) and 1M NaCl extract from nucleus, performed cGAS immunoprecipitation with buffer containing 0.4M NaCl, and then eluted the beads with 1M NaCl buffer. Interestingly, we detected cGAS inhibitory activity from eluates of 1M NaCl extract immunoprecipitation but not eluates of s20 immunoprecipitation (Figure 3-8E). This indicates that we were able to enrich this putative cGAS inhibitor(s) from nuclear extract with immunoprecipitation and high salt (1M NaCl) elution.

We characterized the inhibitor(s) by heating and enzyme digestion tests (Figure 3-8F). We found that this putative inhibitor(s) was resistant to heat inactivation (95 degrees for 5 minutes), and it was also resistant to DNase and RNase treatment. The fact that protease K could kill the inhibitory effect proved that the inhibitory effect came from certain protein component(s).

Conclusions and discussion

In this project, we discovered that instead of simply being in the cytosol, the localization of cGAS changes dynamically during cell cycle. With the majority of cGAS in the nucleus, it is intriguing that cGAS is not activated by chromatin. As previously reported, cytosolic chromatin (Gluck et al. 2017) and chromatin assembled in vitro (Mackenzie et al. 2017) can effectively activate cGAS. So there should be a mechanism to control the activity that cGAS in the nucleus, probably by the existence of an inhibitor that is associated with chromatin. As shown in Figure 3-8A, it is interesting to see that while cGAS in the nucleus bound with chromatin but was not activated, it can still be activated by ISD. This further indicates that the putative cGAS inhibitor is restricted to chromatin structure. In this case, as we added ISD into the reaction, the ISD would compete for cGAS binding with chromatin. When the cGAS protein bound with ISD, it was disassociated from chromatin and thus from the putative inhibitor, and that’s why cGAS could be activated by ISD. In our experiments, we have also found that there were some fundamental biochemical differences between cytosolic cGAS and nuclear cGAS (data not shown), such as that nuclear cGAS could only be soluble in solutions with salt concentration high than about 350mM NaCl and that nuclear cGAS appeared to have much larger molecular weight than cytosolic cGAS, even in buffer with detergent or with 1M NaCl. So it will be interesting to further characterize the nuclear cGAS, which may offered further clues on how cGAS behavior and activity are regulated in the nucleus.

From our observation, it seems that the only way for cGAS to be associated with chromatin/chromosome and enter the nucleus is through cell cycle when nuclear membrane breaks down. Though we do not currently have absolute perfect evidence (that would be to show that point mutations that abolish cGAS DNA binding can prevent cGAS from binding with chromatin/chromosome) to prove that the binding of cGAS and chromatin/chromosome is through DNA and the DNA binding domain on cGAS, it’s very likely to be the case.

Considering the physiological function of cGAS being recruited to the nucleus during cell cycle, there could be multiple possibilities. First of all, it could be a strategy for the cell to avoid unwanted activation of cGAS. During cell cycles, when nuclear membrane breaks down, nuclear chromosomal DNA is exposed to cGAS as potential ligand. In this case, recruiting cGAS to the chromosome and then keep it inactive by certain chromosome binding cGAS inhibitor(s) could be an effective way to control cGAS activity. Another potential role of cGAS in the nucleus could be that cGAS may functions as an important regulator in the cell cycle and cell senescence, as reported by our colleagues and other researchers (Dou et al. 2017, Gluck et al. 2017, Yang et al. 2017). The DNA fragment generated by oxidative stress, telomere shortening, and tumor genomic instability could be sensed by cGAS and initiate responses, including promoting cell senescence. A third possibility is that cGAS may act as a nuclear DNA sensor. As the predominant cytosolic DNA sensor, it has long been proven that it is responsible for eliciting the immune response against DNA virus including Herpes Simplex Virus. During the life cycles of HSV, following the entry of host cells, the viral capsid extrudes its genomic double-stranded DNA directly into the nucleus. It is still debating that how cGAS senses HSV DNA, if it were only functional in the cytosol. One explanation could that in some situations, the HSV particles might be defective and after these particles are taken by macrophages or dendritic cells, their DNA would get exposed in the cytoplasm and thus recognized by cGAS. However, it is also possible that the cGAS in the nucleus, as we observed, could be activated by HSV genomic DNA. It would be interesting to further investigate how cGAS remains inactive while binding with the host chromatin but can be activated by viral DNA or self-generated damage DNA.

Material and methods

Cells and reagents

The THP1-Lucia™ ISG cell line was purchased from InvivoGen (Cat. Code: thpl-isg); Lentiviral infection and establishment of stable cell lines were described previously (Tanaka and Chen 2012); cGAS antibody was from Sigma, MAVS antibody was from Santa Cruz and ikba antibody was from Cell Signaling. ANTI-FLAG® M2 Affinity Gel was from sigma. E. coli Ribosome was purchased from New England BioLabs. Benzonase was purchased from Sigma. RNases was purchased from ThermoFisher.

In vitro cGAMP synthesis assay

In 20 or 40uL reaction mix, the following components were added: 20 mM Tris-HCl (pH 7.5), 5mM MgCl2, 0.2ug/ul BSA, 1mM ATP, 0.5mM GTP and 10ng/uL ISD or HT-DNA. L929 cytosol (s20) or 1.5 or 3ng of recombinant human cGAS was used as source of cGAS activity. The reactions were incubated at 37 degrees for 30 minutes and then 95 degrees to heat inactivate. For reactions with cGAS inhibitor(s), the source of cGAS activity was first incubated with the putative inhibitor(s) for 15 minutes on ice, then DNA/ATP/GFP mix was added to initiate reaction.

Quantification of cGAMP using THP-1 reporter cell

THP1-Lucia™ ISG cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 50uM 2-Mercaptoethanol. For quantification of cGAMP, cells were pelleted and suspended to a final concentration of 5 million/mL in culture medium supplemented with 25ng/mL Perfringolysin O (PFO). Suspended cells were aliquoted as 50ul/well in 96-well cell culture plates and 5.5ul of the heated reaction mix or cGAMP standards. The cells were incubated in cell incubator overnight (at least 12 hours). For each reaction, 12ul of cell culture supernatant was mixed with 40uL of Lucia substrate and the luciferase activity was measured by luminometer.

TLC assay of cGAS activity

Enzyme activity of recombinant full-length cGAS was tested with or without putative inhibitor in buffer containing 20mM Tris-HCl (pH 7.5), 5mM MgCl2, and 0.2ug/mL BSA. cGAS (final concentration around 30nM) or equal volume of water was incubated with activating ligand DNA (final concentration 10ng/ul) in the presence of ATP, GTP, and trace amounts of [α-32P] ATP at 37 degrees for 30 minutes. Reactions were terminated by heating at 95 degrees for 5 minutes. One microliter of each reaction was used for TLC assay. When PEI-Cellulose F TLC plate (EMD Millipore) was used, the reaction products were separated with the use of 5mM NH4HCO3 in 15% H2O and 85% Ethanol as solvent. Plates were dried and radiolabeled products were detected by imaging the exposed phosphor screen using a Typhoon phosphorimager (GE Healthcare).

Cell cycle synchronization

To arrest cells at M phase, split Hela cells by 1:4 one day before and treat the cell with 100ng/ml Nocodazole in the culture medium at following day for 14 hours. The chromosomes were visualized by live-cell staining and imaging using NucBlue™ Live ReadyProbes™ Reagent (ThermoFisher R37605). To arrest cells at G1/S phase, first treat the cells with 2mM Thymidine for 18 hours (cells were arrested at S phase), then remove Thymidine containing medium, wash with PBS, and add fresh culture medium to release cell cycle and culture for 9 hours. After releasing, perform second block by adding 2mM Thymidine for 17 hours. Finally, remove Thymidine, wash with PBS, add back fresh medium and start tracking the synchronized cell cycle progress.

Cell lysis and extraction of different fractions

To separate cytosol protein and cell nucleus, cells was pelleted in tubes and then re-suspended with 3 volumes of hypotonic buffer (10mM Tris-HCl (pH 7.5), 10mM KCl, 3mM MgCl2) supplemented with cOmplete™ Protease Inhibitor Cocktail (Roche). The cells were lysed either by douncing or by going through gauge 30 needles for 5 times. Then spin down at 5,000g for 10 minutes. The supernatant (s5) was further subjected to 20,000g to get cytosolic proteins (s20), the pellet (p5) was extensively washed by hypotonic buffer and labeled as nucleus. For cGAS sequential extraction, the hypotonic buffer lysate was subject to 20,000g centrifugation, the supernatant (s20) was labeled as cytosol and the pellet was suspended and extracted with RIPA buffer. After spin down, the supernatant was labeled and RIPA\_extract and the pellet was boiled in 2x SDS loading buffer for subsequent analysis.

Immunoprecipitation and elution

Cells were collected as cell pellet and lysed in hypotonic buffer supplemented with 0.5% NP-40, the lysate was subjected to 5,000g centrifugation and the pellet (p5, labeled was nucleus) was washed and finally suspended in 1x volume of hypotonic buffer. The nucleus suspension was treated by Benzonase on ice for 30 minutes and then washed extensively using hypotonic buffer to remove residual Benzonase. Then hypotonic buffer supplemented with 1M NaCl was used to extract nuclear protein from the Benzonase digested nucleus (labeled as 1M NaCl extract). After extraction, the 1M NaCl extract was diluted with hypotonic buffer to reach a final salt concentration of 0.4M or 1M. Then ANTI-FLAG® M2 Affinity Gel was added to the diluted extract for immunoprecipitation at 4 degrees for 6 hours to overnight. After immunoprecipitation, the beads were washed with hypotonic buffer supplemented with 0.4M NaCl for 5 times. Then elution buffer (50mM Tris-HCl (pH7.5), 10mM KCl, 10% Glycerol, 0.5mM DTT, and 0.1ug/ul BSA) supplemented with 1M NaCl or Flag peptide was used to elute proteins from the beads.