Attenuation of cGAS/STING Activity During Mitosis

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The innate immune system recognizes cytosolic DNA associated with microbial infections or cellular stress via the cGAS/STING pathway, leading to activation of phospho-IRF3 and downstream IFN-I and senescence responses. To prevent hyperactivation, cGAS/STING is presumed to be non-responsive to chromosomal self DNA during open mitosis, though specific regulatory mechanisms are lacking. Given a role for the Golgi in STING activation, we investigated the state of the cGAS/STING pathway in interphase cells with artificially vesiculated Golgi and in cells arrested in mitosis. We find that while cGAS activity is impaired through interaction with mitotic chromosomes, Golgi integrity has little effect on the enzyme's production of cGAMP. In contrast, STING activation in response to either foreign DNA (cGAS-dependent) or exogenous cGAMP is impaired by a vesiculated Golgi. Overall our data suggest a secondary means for cells to limit potentially harmful cGAS/STING responses during open mitosis via natural Golgi vesiculation.

Introduction

Cells possess intrinsic sensory pathways as part of the innate immune system to detect microbial infection or other physiological insults [1]. Foreign nucleic acids are often recognized as pathogen-associated molecular patterns (PAMPs) through a number of pattern recognition receptors (PRRs) [2], causing activation of NFκB-dependent inflammatory cytokine responses and/or IRF3/7-dependent type-I interferon (IFN-I) responses [1, 3]. The cGAS/STING pathway is recognized as a central component of innate immunity for cytosolic DNA recognition and downstream IFN-I responses [4-8]. Cytosolic DNA is recognized by the enzyme cGAS, triggering production of the cyclic dinucleotide 2',3'-cGAMP [9]. STING, a transmembrane endoplasmic reticulum (ER) protein [10, 11], is activated by direct binding to cGAMP [12].

Upon activation by cGAMP at the ER, dimeric STING undergoes a conformational change [13] and traffics to the Golgi, a prerequisite for assembly of the STING/TBK1/IRF3 complex and downstream IFN-I responses [14]. cGAMP-dependent STING recruitment of TBK1 [15] can lead to phosphorylation of IRF3 and NFκB, stimulating both IFN-I and proinflammatory cytokine responses [10, 16, 17]. Trafficking of STING to the Golgi is regulated by several host factors including iRHOM2-recruited TRAPß [18], TMED2 [19], STIM1 [20], TMEM203 [21], and Atg9a [22]. STING activation at the Golgi requires palmitoylation [23] and ubiquitylation [24, 25], allowing for assembly of oligomeric STING and recruitment of TBK1 and IRF3 [26-28]. STING also interacts with the ER adaptor SCAP at the Golgi to facilitate recruitment of IRF3 [29],

In addition to innate defense against microbial infections, cGAS/STING is involved in cellular responses to DNA damage and replicative/mitotic stress [5, 30-36]. DNA damage, replicative stress, chromosomal instability, and mitotic errors can lead to the formation of micronuclei which can trigger antiproliferative IFN-I and senescence responses via cGAS/STING [37].

Unabated activation of cGAS/STING can lead to harmful autoinflammatory and senescence responses, exemplified by type-I interferonopathies associated with mutations in STING [38-41] or mutations in the DNases TREX1 and DNASE2 that normally clear cells of cGAS-stimulatory DNA [42-45]. Given the harmful effects of cGAS/STING hyperactivation, cells need regulatory mechanisms to avoid self-stimulation of cGAS/STING during mitosis. Cytosolic compartmentalization of cGAS was initially proposed as a mechanism, but nuclear chromosomes and cytosolic compartments mix upon mitotic nuclear envelope breakdown (NEBD), suggesting a more elaborate means of cGAS/STING attentuation during cell division.

The chromatinized nature of cellular genomic DNA has been proposed to mitigate cGAS/STING activity, with histones structurally marking DNA as "self." This is an attractive model as many DNA viruses sensed by cGAS/STING upon initial entry, trafficking,

and uncoating (prior to viral DNA replication) contain either naked, unchromatinized dsDNA (e.g. herpesviridae [46, 47]) or DNA that is packaged with non-histone viral core proteins (e.g. adenoviridae, poxviridae, and asfarviridae [48-52]).

Since cGAS localizes to condensed chromosomes upon NEBD [35, 53], others have asked whether cGAS is activated by chromosomes, and if not, what mechanisms exist to prevent such self-activation. Recent studies have revealed that i) chromosome-bound cGAS is tightly tethered to chromatin, potentially via interactions with H2a/H2b dimers, ii) chromatin interaction does not involve the DNA-binding domains of cGAS required for "typical" activation by dsDNA, and iii) that chromosome binding results only in weak activation of cGAS with relatively low production of cGAMP [34, 53, 54].

Given the importance of the Golgi in STING-dependent activation of IRF3, we hypothesize a parallel mechanism for cGAS/STING regulation during open mitosis – Golgi vesiculation [55, 56]. Here, we find that chemical dispersal of the Golgi abrogates cGAS/STING-dependent phospho-IRF3 responses to transfected DNA. Furthermore, we show that cGAS/STING activity in response to transfected DNA is diminished during open mitosis, correlating with the vesiculated state of the mitotic Golgi. This Golgi-dependent weakening of cGAS/STING responses to foreign DNA occurs at the level of STING, as cGAS activity is downregulated upon mitotic chromosome binding but largely unaffected by Golgi integrity. The vesiculated state of the mitotic Golgi may therefore provide an additional safeguard mechanism, ensuring that potentially harmful cGAS/STING responses to self-DNA are minimized during cell division.

Results & Discussion

Human Keratinocytes Respond to Foreign DNA via cGAS/STING

We chose to investigate activity of the cGAS/STING pathway in HaCaTs, a spontaneously immortalized human keratinocyte cell line [57]. This cell line is a good model for primary keratinocytes that serve a barrier function and as host cells for a number of viral infections including papillomaviruses, herpesviruses, mosquito-transmitted alphaviruses and flaviviruses [58-64], and numerous bacterial and fungal pathogens [65, 66]. As not all cell types express every PRR, we sought to ensure the cGAS/STING pathway was functional in HaCaTs and that this pathway was the primary mode of IRF3 phosphorylation in response to foreign DNA.

HaCaTs were transfected with siRNAs targeting the cGAS/STING pathway and subsequently transfected with endotoxin-free dsDNA plasmid pGL3 (Fig. 1). IRF3 was phosphorylated in response to DNA transfection, exemplifying the ability of exogenous dsDNA to activate the cGAS/STING pathway in HaCaTs**.** IRF3 phosphorylation was impaired when pGL3 was transfected following siRNA knockdown of cGAS, STING, or IRF3, indicating HaCaTs utilize the cGAS/STING pathway as the primary mechanism of activating a phospho-IRF3 response to foreign DNA.

Artificial Golgi Vesiculation Impairs cGAS/STING Activity at the Level of STING

Activated STING traffics to the Golgi to oligomerize and complex with TBK1/IRF3. Since Golgi trafficking is critical for STING/TBK1/IRF3 complex assembly and activation, we hypothesized that a vesiculated Golgi would prevent cGAS/STING from responding to foreign DNA. To test this idea, we used golgicide A (GCA), a selective and reversible inhibitor of the Arf1 guanine nucleotide exchange factor GBF1 [67]. GCA treatment completely vesiculated the Golgi apparatus (Fig. 2E), mimicking the dispersed mitotic Golgi. As expected, IRF3 phosphorylation was upregulated upon transfection of pGL3 in HaCaTs with intact Golgi; however, GCA-mediated Golgi vesiculation impaired DNA-dependent IRF3 phosphorylation (Fig. 2A, B).

The RIG-I-like receptors (RLR) recognize intracellular viral RNA products through the mitochondria-resident adaptor protein MAVS to elicit NFκB inflammatory and IRF3/7-dependent IFN-I responses [68, 69]. Transfection of the dsRNA analog poly(I:C) elicited a phospho-IRF3 response regardless of GCA treatment (Fig. 2A, C), suggesting that the cGAS/STING pathway, but not the RLR pathway, is regulated by Golgi morphology. GCA had no effect on cGAMP production in response to pGL3 transfection (Fig. 2D), indicating that despite equivalent transfection, Golgi dispersal blocked the pathway downstream of cGAS.

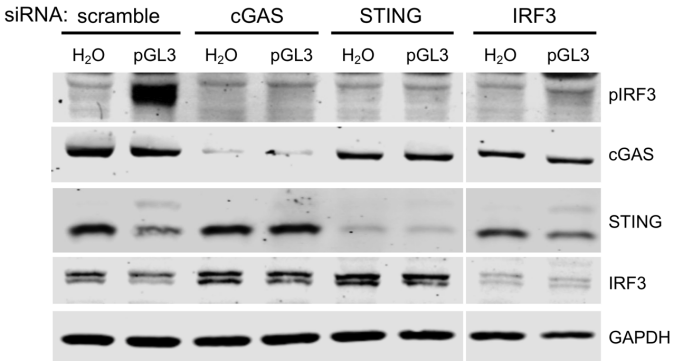

Figure 1. *Human Keratinocytes Respond to Foreign DNA via cGAS/STING*. HaCaTs were transfected with siRNAs for 16 hr, followed by transfection with 500 ng pGL3 or water for 90 min.

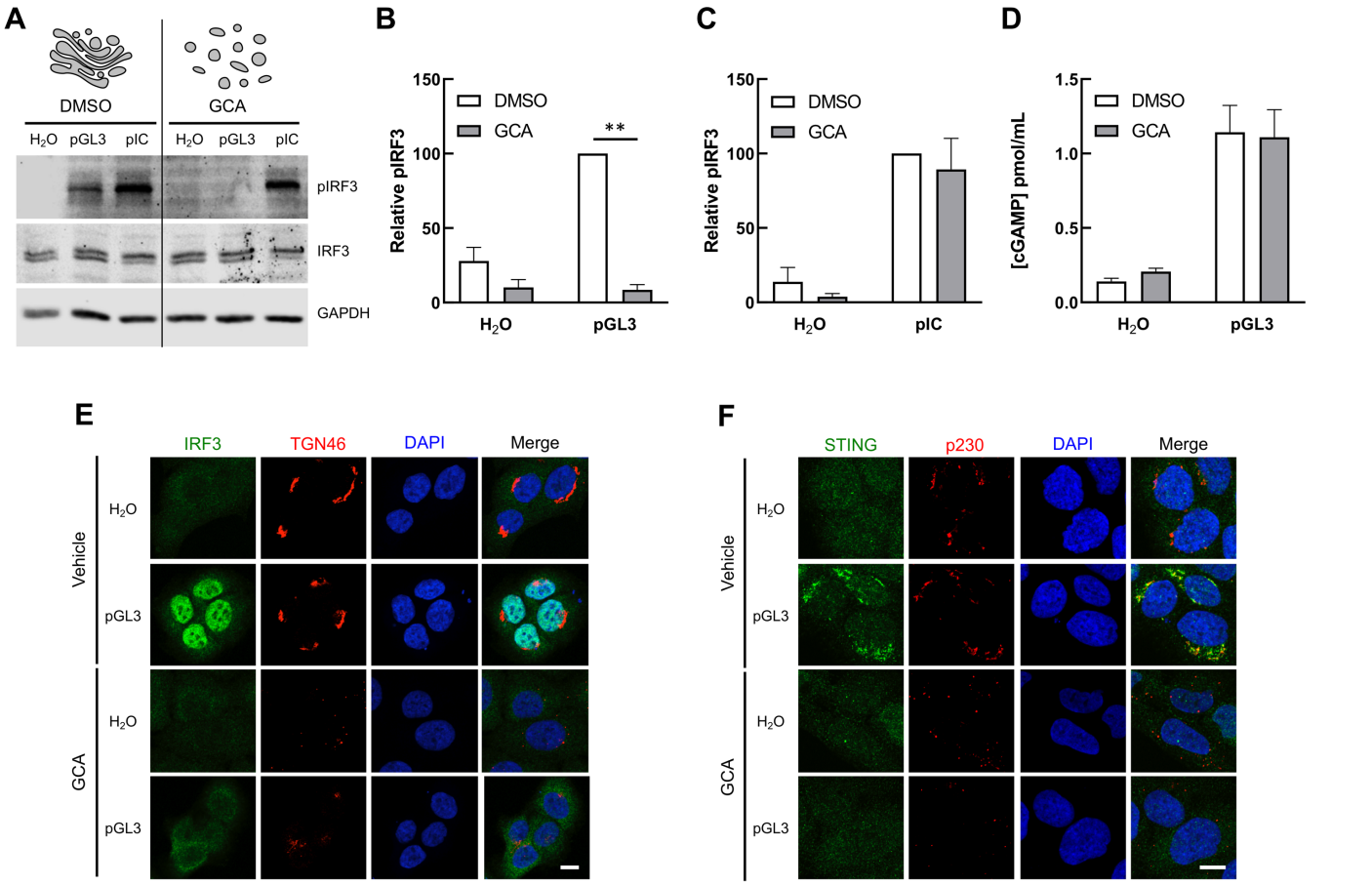

Figure 2. *Artificial Golgi Vesiculation Impairs cGAS/STING Activity at the Level of STING*. cGAS/STING activation in human keratinocytes is impaired by Golgi vesiculation. Cells were treated with Golgicide A (GCA) or vehicle for 1 hr prior to a 90 min nucleic acid transfection. **(A)** Nucleic acid transfection elicited IRF3 phosphorylation in vehicle-treated cells, but GCA impaired DNA-dependent phosphorylation of IRF3, $^{**}p < 0.001$, n = 5. **(B)** cGAMP production in GCA and vehicle-treated cells was similar, n = 6. **(C, D)** GCA completely vesiculated the Golgi and impaired IRF3 nuclear translocation **(C)** and STING localization to the Golgi **(D)**. Scale bars = 10 μm.

We further examined the effects of Golgi vesiculation on cGAS/STING activity via confocal microscopy. In vehicle-treated HaCaTs, IRF3 (Fig. 2E) and STING (Fig. 2F) were distributed throughout the cell. pGL3 transfection resulted in nuclear accumulation of IRF3 (Fig. 2E) and the accumulation of STING at p230-positive Golgi structures (Fig. 2F), indicating pathway activation. In the presence of GCA, STING was scattered (Fig. 2F) and IRF3 remained cytosolic (Fig. 2E) upon pGL3 transfection. Overall, these data show that cGAS/STING activity is blunted at the level of STING upon Golgi vesiculation, similar to what may occur during mitosis.

cGAS/STING Activity is Attenuated During Mitosis

We next investigated the impact of natural mitotic Golgi vesiculation on the ability of cGAS/STING to sense and respond to exogenous DNA. Secretory ER to Golgi traffic is blocked during mitosis [70-73]. Golgi integrity is dependent on cargo transport from ER-exit sites (ERES), and mitotic arrest of COPII-dependent ERES traffic causes Golgi dispersal [74, 75]. As assembly of the activated STING/TBK1/IRF3 complex requires STING transport from ERES to the Golgi [14], we hypothesized that mitotic Golgi dispersal and inactivation of ERES would blunt cGAS/STING responses.

We devised a method to synchronize cells at prometaphase (Fig. 3A). Briefly, cells were cultured at 100% confluence for 48 hr in 1% serum, causing quiescent arrest in G_0. Cells were released by replating in 10% serum, allowing for G_1 reentry and progression to S. Twenty-four hours post-G_0 release, cells were synchronized at prometaphase with low dose nocodazole (NOC) for 12 hr. Upon NOC washout, synchronized cells progressed through mitosis, returning to G_1 within 3 hr. Propidium iodide staining showed cells enriched at G_2/M after NOC treatment, and the majority of cells back in G_1 by 3 hr post-NOC release (Fig. 3A). Phosphorylated histone H3 (pH3) was enriched in NOC-synched cells, decreasing as cells returned to G_1 (Fig. 3B).

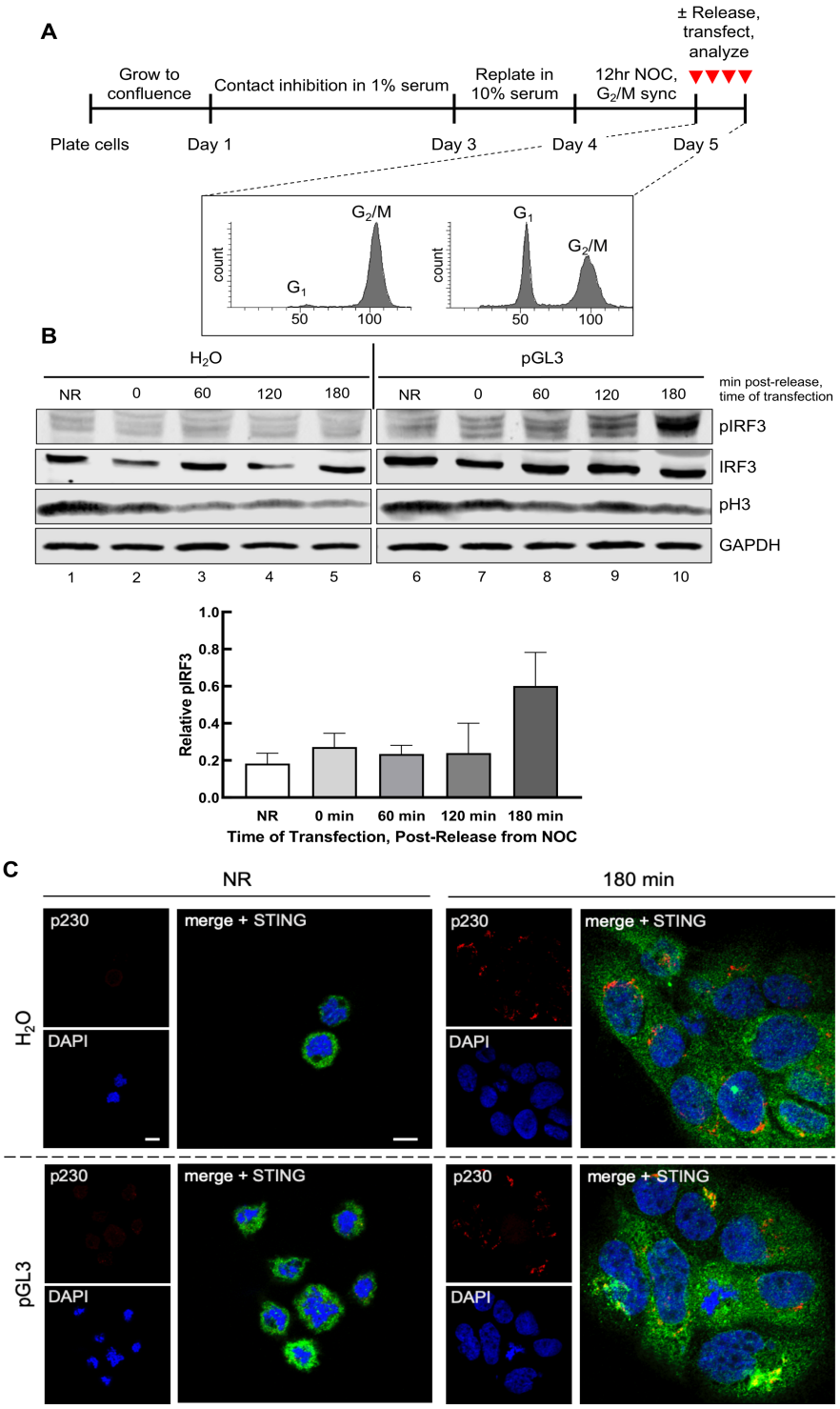

Figure 3. *cGAS/STING Activity is Attenuated During Mitosis.* **(A)** Cell synchronization method. Cells were pre-synchronized in G_1 by contact inhibition and growth in low serum. Cells were then released by replating at subconfluence in 10% serum and synchronized in prometaphase with NOC. Cell cycle analysis by PI-staining. Cells were transfected with 500 ng pGL3 for 90 min at various times post-release from NOC. **(B)** pIRF3 activation in response to pGL3 was dampened when arrested cells were transfected, gradually returning as cells completed mitosis and returned to interphase, n = 4. **(C)** Golgi localization of STING was impaired when arrested cells (NR) were transfected, but was restored upon return to interphase (180 min). Scale bars = 10 μm.

We used this scheme to assess mitotic cGAS/STING responses to pGL3 transfection. Cells were transfected following the 12 hr NOC sync (non-released group, NR), or at 0, 60, 120, or 180 min post-release, and cGAS/STING activity was assessed 90 min post-transfection. Cells arrested at prometaphase mounted a very weak phospho-IRF3 response to pGL3 transfection compared to cells which had returned to G_1 after a 3 hr release (Fig. 3B, compare lanes 6 to 10). These arrested cells had condensed chromosomes with dispersed Golgi (Fig. 3C, NR). STING had a cytosolic but granular distribution in arrested cells, which did not change upon pGL3 transfection. In contrast, STING clearly localized to Golgi structures upon pGL3 transfection of interphase cells (Fig. 3C, 180 min). On average, across four independent synchronization experiments, pGL3-dependent phosphorylation of IRF3 was attenuated during mitosis, only becoming robust at 180 min (Fig. 3B), when the bulk population of cells reached G_1 and had intact Golgi with clear STING localization upon pGL3 transfection (Fig. 3C, 180 min).

cGAS and STING are Non-Responsive During Mitosis

Recent work has shown that upon NEBD, cGAS localizes to mitotic chromosomes via direct binding to H2a/H2b dimers [53]. However this binding is not via the DNA-binding domains of cGAS that underlie DNA-dependent activation, resulting in a relatively low production of cGAMP. Thus, chromatin appears to blunt cGAS activation [34, 53], suggesting a means for the cell to avoid cGAS-driven IFN responses to self chromosomes during open mitosis. One unexplored aspect is whether chromatin-bound cGAS, or the pool of cGAS that might remain cytosolic during open mitosis, would still be responsive to foreign (naked) DNA–and whether that activation would then cause IRF3 phosphorylation.

We observed only low activation of IRF3 in response to pGL3 transfection

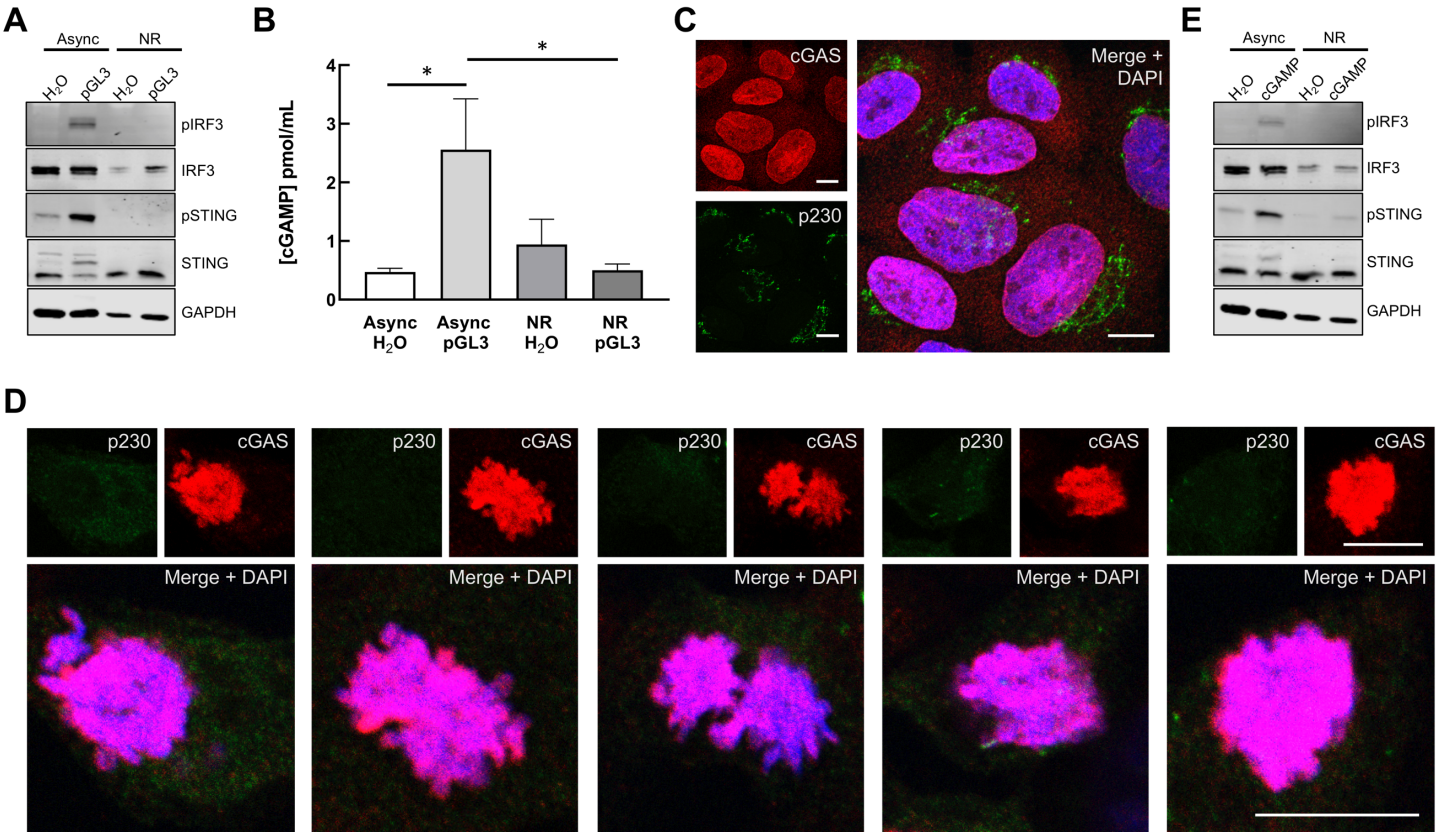

Figure 4. *cGAS and STING are Non-Responsive During Mitosis.* **(A)** Mitotic phospho-IRF3 and phospho-STING responses to pGL3. Asynchronous and arrested cells were stimulated by transfection of 500 ng pGL3 or water for 90 min prior to western blots. **(B)** Mitotic cGAMP responses to pGL3. Asynchronous and arrested cells were transfected with 500 ng pGL3. Non-internalized transfection complexes were removed by media change 2 hr after the initial transfection. Five hours post-transfection, cGAMP levels were measured by ELISA. *$p < 0.05$, $n = 4$. **(C, D)** cGAS subcellular localization and Golgi morphology in asynchronous **(C)** and arrested **(D)** HaCaT cells. Scale bars = 10 μm. **(E)** Mitotic phospho-IRF3 and phospho-STING responses to exogenous cGAMP. Asynchronous and arrested cells were treated with 25 μg/mL 2'-3'cGAMP for 2 hr prior to western blots.

in our arrested cells (Fig. 3B and 4A). To determine if the dampening of the pathway in these arrested cells was at the level of cGAS or STING, we measured cGAMP production in response to pGL3 transfection and examined cGAS subcellular distribution. Similar to phospho-IRF3, cGAMP production was blunted in transfected arrested cells (NR) compared to asynchronous interphase cells (Fig. 4B). Confocal microscopy of asynchronous cells revealed that cGAS was mostly nuclear although signal was evident within the cytosol (Fig. 4C), in agreement with a recent report [54]. Within arrested cells, the vast majority of cGAS was chromatin-bound and Golgi were well dispersed (Fig. 4D). These results agree with recent work showing that cGAS is predominantely nuclear, regardless of cell cycle phase or activation status [54]. Combined, our data suggest that chromatin-bound cGAS is unable to produce a robust cGAMP response to either chromosomes or transfected DNA.

Although we detected slightly elevated levels of cGAMP in unstimulated arrested versus asynchronous cells (Fig. 4B), the subtle difference was not significant, consistent with recent work showing cGAS generates only low levels of cGAMP upon activation by chromatin [34, 53]. Mitotic cells could potentially take up exogenous cGAMP via the SLC19A1 transporter [76, 77] or directly from neighboring cells [78, 79]. Recent work has revealed a primordial role for cGAMP-dependent STING activation in triggering autophagy through WIPI2/ATG5 in a TBK1/IRF3-independent manner [80]. These Golgi-derived autophagosomes promote clearance of cytosolic DNA and incoming DNA viruses like HSV1 [80]. Although mitotic cells appear to avoid classical induction of autophagy via CDK1 phosphorylation of ATG13, ULK1, and ATG14 [81, 82], downstream STING-dependent activation of autophagophore formation/elongation via WIPI2/ATG5 during open mitosis could be deleterious to daughter cell survival [83, 84].

Considering the potential for activation of STING by low levels of cGAMP, we assessed whether mitotic Golgi vesiculation would prevent STING activation by exogenous cGAMP. Neither transfection of pGL3 nor addition of exogenous cGAMP stimulated

phospho-IRF3 or phospho-STING in arrested cells (Fig. 4E), suggesting that Golgi vesiculation reinforces cGAS inactivation as a secondary barrier to cGAS/STING activation during open mitosis.

Given that cGAS/STING-dependent elevation of phospo-IRF3 during mitosis (particularly during prolonged mitosis) can induce apoptosis [53] and activated STING can induce a potentially harmful autophagic response [80], a parallel dampening mechanism like Golgi dispersal likely serves an important role to limit potentially harmful cGAS/STING signaling during open mitosis. Further, many other viruses and bacteria induce dramatic alteration of Golgi membranes during infection [85-89]. Microbial alteration of Golgi integrity may be an unrecognized tactic to blunt host cGAS/STING responses.

Materials & Methods

Tissue Culture

HaCaT cells were grown in high glucose DMEM supplemented with 10% FBS and Ab/Am. Cells were maintained at 37°C with 5% CO_2 and passaged every 2–3 days.

Nucleic Acid Transfections

HaCaTs were plated at 60,000 cells per well in a 24-well plate. Cells were transfected with 500 ng endotoxin-free pGL3 (5.3 kb) or 500 ng high molecular weight poly(I:C) (InvivoGen tlrl-pic, 1.5 kb to 8 kb) using Lipofectamine 2000 (ThermoFisher 11668) in OptiMEM (Life Technologies). At various timepoints post-transfection, cells were washed once with PBS and lysed in 1x RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS), supplemented with 1x reducing SDS-PAGE loading buffer, 1x protease inhibitor cocktail (Sigma P1860), 1 mM PMSF, and 1x PhosSTOP phosphatase inhibitor cocktail (Roche 04906845001). Samples were boiled for 5 min at 95°C and stored at -80°C until processed by gel electrophoresis.

siRNA Experiments

Pooled scramble (sc-37007), cGAS (sc-95512), STING (sc-92042), and IRF3 (sc-35710) siRNA duplexes were obtained from Santa Cruz Biotechnologies. HaCaTs were plated at 30,000 cells per well in a 24-well plate with Ab/Am-free DMEM/10% FBS. The following day, cells were washed twice with PBS and the PBS replaced with OptiMEM. Cells were transfected with 50 nM siRNA using Lipofectamine RNAiMax (Life Technologies 13778150). At 16 hr post-siRNA transfection, cells were washed twice with PBS and the PBS was replaced with Ab/Am-free DMEM/10% FBS. Cells were transfected with pGL3 24 hr post-siRNA transfection, as described above. At 90 min post-transfection, samples were collected for western blotting as described above.

Cell Synchronization

HaCaTs were plated at 7.5 million cells on a 10 cm dish in 10% FBS/DMEM and allowed to reach 100% confluence. The day after plating, the media was changed to 1% FBS/DMEM and cells remained under contact inhibition at low-serum conditions for 48 hr to synchronize at G_0. Cells were then replated at 30,000 cells per well on 24-well plates with or without glass cloverslips in 10% FBS/DMEM and allowed to progress through S phase. After 24 hr, cells were treated with 50 ng/mL nocodazole (Santa Cruz sc-3518) for 12 hr to synchronize at prometaphase, at which point they were released with two PBS washes and incubated in 10% FBS/DMEM. Cells were transfected with pGL3 as described above, and either harvested for western blotting or prepared for immunofluorescence or cell cycle analysis as described.

Cell Cycle Analysis

Cell cycle status was analyzed by propidium iodide (PI) incorporation and flow cytometry. HaCaTs synchronized in the manner described above. At various time points during the sync and release from prometaphase, cells were collected by trypsinization and pelleted at 1000 rpm for 10 min at 4°C. The pellet was resuspended in ice cold 70% ethanol to fix the cells and stored at -20°C until ready for PI staining. Cells were pelleted at 2000 rpm for 15 min at 4°C, resuspended in PBS, pH 7.4 containing 40 µg/mL PI and 500 µg/mL RNase A, and incubated at 37°C for 30 min. PI-stained cells were analyzed using the BD Biosciences FACSCanto-II flow cytometer and Diva 8.0 software.

Immunofluorescence

For Golgi disruption experiments: HaCaTs were plated at 60,000 cells per well on glass coverslips in 24-well plates. The following day, cells were treated with 10 µM Golgicide A (GCA) or DMSO vehicle for 1 hr prior to a 90 min 500 ng pGL3 or poly(I:C) transfection. For mitotic sync experiments: HaCaTs were synced to pro-metaphase as described above. For all experiments, following transfection, cells were fixed with 2% paraformaldehyde/PBS for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100/PBS for 10 min at RT. Samples were blocked in 4% BSA/1% goat serum/PBS overnight at 4°C. Rabbit polyclonal anti-TGN46 (Sigma T7576. 1:500), mouse monoclonal anti-p230 (BD Biosciences 611280,

1:500), mouse monoclonal anti-IRF3 (Abcam ab50772, 1:100), rabbit anti-cGAS (Cell Signaling 15102, 1:100), and rabbit monoclonal anti-STING (Abcam ab181125, 1:500) were used as primary antibodies. Alexa Fluor-488, -555 and -647 labeled goat anti-mouse and goat anti-rabbit secondary antibodies (Life Technologies A11029, A21424, A21429 and A21236) were used at 1:1,000. Samples were then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich D9542-10MG) at 1μg/mL for 30 sec. Coverslips were mounted on glass slides with Prolong Antifade Diamond (Life Technologies P36970) and analyzed by confocal microscopy.

Confocal Microscopy
Following the preparation of immunofluorescence slides, confocal microscopy was performed using a Zeiss LSM510 META system with a 405 nm laser diode, a 488 nm argon laser, and a 543 nm He/Ne1 laser or the Zeiss LSM880 system with 405 nm, 488 nm, 543 nm and 633 nm lasers. Samples were examined using an oiled 63x objective, and Z-stacks with a 0.32 µm depth per plane were taken of each image. Representative single-plane images were processed with the Zen Blue software.

cGAMP Stimulation
HaCaTs were plated at 60,000 asynchronous cells or 130,000 synchronous cells per well in 24-well plates. Cells were treated with 25 µg/mL 2'-3'cGAMP (Invivogen tlrl-nacga23) for 2 hr, then harvested for western blotting as described above.

SDS-PAGE & Western Blotting
Samples were resolved by SDS-PAGE and transferred onto a 0.45 µm nitrocellulose membrane. Rabbit monoclonal anti-GAPDH (Cell Signaling 2118, 1:5000), mouse monoclonal anti-IRF3 (Abcam 50772, 1:100), and rabbit monoclonal anti-STING (Cell Signaling 13647, 1:1000) blots were blocked in 5% non-fat powdered milk dissolved in Tris-buffered saline containing 0.1% Tween (TBST). Rabbit monoclonal anti-phospho-IRF3 (Cell Signaling 4947, 1:1000), rabbit monoclonal anti-phospho-STING (Cell Signaling 19781, 1:1000), and rabbit monoclonal anti-phospho-H3 (Cell Signaling 3377, 1:10,000) blots were blocked in 100% Odyssey blocking buffer (Licor 927–40000). Goat anti-rabbit DyLight 680 (Pierce 35568), goat anti-mouse DyLight 680 (Pierce 35518), goat anti-rabbit DyLight 800 (Pierce 535571) and goat anti-mouse DyLight 800 (Pierce 35521) were used as secondary antibodies at 1:10,000 in either 50% Odyssey blocking buffer/TBST or 5% milk/TBST. Blots were imaged with the Licor Odyssey Infrared Imaging System. Band intensities were quantified by densitometry using ImageJ v1.52a [90].

cGAMP ELISA
Following Golgi vesiculation and mitotic sync experiments, cells were washed 1x with PBS and prepared for cGAMP quantification by the 2′,3′-Cyclic GAMP Direct ELISA Kit (Arbor Assays K067-H1), following the manufacturer's protocol. cGAMP concentrations were normalized with by total protein concentration, as determined by a BCA Assay (Thermofisher 23225).

Statistics
Statistical analyses were performed using Prism 6 (GraphPad Software). Statistics for the Golgi vesiculation experiments were determined by an unpaired t-test with the Holm-Sidak correction. Statistics for the cGAMP ELISAs were determined by ordinary one-way ANOVAs with the Tukey correction for multiple comparisons.

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Competing Interests

The authors have no competing interests to declare.

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