

1 **Innate Immune Priming by cGAS as a Preparatory Countermeasure Against**
2 **RNA Virus Infection**

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17 Running Header: cGAS primes restriction of RNA viruses

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26 **Abstract**

27 The detection of nucleic acids by pattern recognition receptors is an ancient and
28 conserved component of the innate immune system. Notably, RNA virus genomes
29 are sensed by mammalian cytosolic RIG-I-like receptors, thereby activating
30 interferon-stimulated gene (ISG) expression to restrict viral replication. However,
31 recent evidence indicates that the cGAS-STING DNA sensing pathway also
32 protects against RNA viruses. So far, the mechanisms responsible for DNA sensing
33 of RNA viruses, which replicate without known DNA intermediates, remain unclear.
34 By using cGAS gene knockout and reconstitution in human and mouse cell
35 cultures, we discovered that DNA sensing and cGAMP synthase activities are
36 required for cGAS-mediated restriction of vesicular stomatitis virus and Sindbis
37 virus. The level of cGAMP produced in response to RNA virus infection was below
38 the threshold of detection, suggesting that only transient and/or low levels of
39 cGAMP are produced during RNA virus infections. To clarify the DNA ligands that
40 activate cGAS activity, we confirmed that cGAS binds mitochondrial DNA in the
41 cytosol of both uninfected and infected cells; however, the amount of
42 cGAS-associated mitochondrial DNA did not change in response to virus infection.
43 Rather, a variety of pre-existing cytosolic DNAs, including mitochondrial DNA and
44 endogenous cDNAs, may serve as stimuli for basal cGAS activation. Importantly,
45 cGAS knockout and reconstitution experiments demonstrated that cGAS drives
46 low-level ISG expression at steady state. We propose that cGAS-STING restricts
47 RNA viruses by promoting a preparatory immune activation state within cells, likely
48 primed by endogenous cellular DNA ligands.

49 **Importance**

50 Many medically important RNA viruses are restricted by the cGAS-STING
51 DNA-sensing pathway of innate immune activation. Since these viruses replicate
52 without DNA intermediates, it is unclear what DNA ligand(s) are responsible for
53 triggering this pathway. We show here that cGAS's DNA binding and signaling
54 activities are required for RNA virus restriction, similar to the mechanisms by which it
55 restricts DNA viruses. Furthermore, we confirmed that cGAS continuously binds host
56 DNA, which was unaffected by RNA virus infection. Finally, cGAS expression
57 correlated with the low-level expression of interferon-stimulated genes in uninfected
58 cells, both *in vitro* and *in vivo*. We propose that cGAS-mediated sensing of
59 endogenous DNA ligands contributes to RNA virus restriction by establishing a
60 baseline of innate immune activation.

61 **Introduction**

62 A key feature of innate immunity is the detection of pathogen-associated
63 molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (1). For
64 mammalian cells, viral nucleic acids are detected by distinct PRRs, triggering
65 interferon-stimulated gene (ISG) expression to set up an antiviral state. During RNA
66 virus infections, uncapped and double-stranded RNAs are detected in the cytosol by
67 the PRRs retinoic acid-inducible gene I (RIG-I) and related RIG-I-like receptors
68 (RLRs). However, the recent discovery of the cGAS-STING cytosolic DNA sensing
69 pathway, and the observation that it can also restrict RNA viruses (2), reveals a need
70 to further investigate the mechanisms of nucleic acid sensing during RNA virus
71 infection.

72 The stimulator of interferon genes (STING) is an endoplasmic reticulum- and
73 mitochondrial-bound protein that spontaneously activates ISG expression when
74 overexpressed (2). Although STING is involved in DNA sensing, STING^{-/-} mice and
75 mouse endothelial fibroblasts (MEFs) are more permissive for vesicular stomatitis
76 virus (VSV), a negative-strand RNA virus (2, 3). Additionally, studies in MEFs
77 deficient in three prime repair exonuclease 1 (TREX1), a nuclease important for the
78 turnover of cytosolic retroelement cDNAs (4), have described enhanced antiviral
79 phenotypes in response to a wide array of RNA viruses and retroviruses,
80 presumably due to the accumulation of DNA in the cytosol (5, 6). It appears that this
81 DNA-based restriction is broad, as many RNA viruses have evolved mechanisms to
82 subvert the cGAS-STING pathway, including flaviviruses (7-9), hepaciviruses (10,
83 11), picornaviruses (3), coronaviruses (12-17), and influenza A virus (18).

84 STING does not directly interact with cytosolic DNA, but functions as an innate
85 immune adaptor protein to transduce signals between cyclic GMP-AMP synthase
86 (cGAS) and Tank-binding kinase 1, which subsequently phosphorylates the
87 transcription factor interferon regulatory factor 3 (IRF3) to initiate an ISG response
88 (19). Recent evidence also suggests that STING inhibits translation by unknown

89 mechanisms and may restrict RNA virus replication independent of IRF3 activation
90 (20).

91 cGAS is a nucleic acid-binding protein specific for dsDNA and DNA:RNA hybrids
92 that also has nucleotidyl transferase activity (21-24). DNA binding induces structural
93 changes to form the cGAS active site, which synthesizes a non-canonical 5'-2'- and
94 5'-3'-linked cyclic dinucleotide known as cyclic guanosine monophosphate–
95 adenosine monophosphate (cGAMP) (25-28). cGAMP is a diffusible secondary
96 messenger that specifically binds to STING with high affinity ($K_D \sim 4$ nM), thereby
97 inducing a downstream innate immune response (29-32).

98 For RNA viruses that replicate in the cytosol without a DNA intermediate, the
99 specific ligands that activate cGAS remain unclear. At present, the prevailing
100 hypothesis is that RNA viruses induce release of mitochondrial DNA (mtDNA) into
101 the cytosol, thereby activating innate immune responses (7, 33-36). However, it is
102 unclear whether mitochondrial damage is a conserved feature of RNA virus
103 infection, nor is it clear that cGAS-STING activation follows the same pathway for
104 both RNA and DNA viruses.

105 In this study, we investigated whether the DNA binding and cGAMP synthesis
106 activities of human cGAS (hcGAS) are required for RNA virus restriction. While both
107 activities were required, the amount of cGAMP produced during virus infection was
108 too low to detect. We also confirmed that hcGAS binds mtDNA in both uninfected
109 and infected cells but did not observe increased cytosolic or cGAS-associated
110 mtDNA in response to RNA virus infection. We found that cGAS stimulated
111 smoldering, low-level innate immune activation, most likely in response to
112 endogenous DNA ligands, suggesting that cGAS-STING can passively restrict
113 incoming RNA viruses.

114 **Results**

115 **cGAS mediates restriction of RNA viruses in immortalized MEFs.** To clarify
116 the role of cGAS in restriction of RNA virus replication, we performed viral
117 single-step growth curve experiments in wild-type (WT) and cGAS^{-/-} (KO) MEFs
118 immortalized with SV40 large T antigen (Figure 1). Both VSV-GFP and SINV-GFP
119 grew to higher titers in KO MEFs (Figure 1A, B). We then asked whether
120 reconstituting cGAS expression in KO MEFs could restore RNA virus restriction by
121 performing VSV plaque assays on WT MEFs, KO MEFs, or KO MEFs stably
122 expressing hcGAS-HA3x, a functional, triple HA-tagged form of human cGAS (34).
123 As seen in Figure 1C, both WT and hcGAS-reconstituted (KO+WT) cells significantly
124 reduced VSV-GFP plaque formation compared to KO MEFs. These results confirm
125 previous observations that cGAS can restrict RNA virus infection.

126 **The cGAS DNA binding- and cGAMP synthase active site residues are**
127 **essential for RNA virus restriction.** It is currently unclear whether cGAS restricts
128 RNA viruses via the same mechanism that it restricts DNA viruses. We therefore
129 asked whether the DNA binding and cGAMP synthase activities, which are required
130 for DNA sensing and downstream STING activation, are also required for
131 cGAS-mediated restriction of RNA viruses. Specifically, we reconstructed previously
132 described loss-of-function mutations in the DNA binding pocket and cGAMP
133 synthase active site within hcGAS-HA3x (27) (Figures 2A and S1), then restored
134 cGAS expression in KO MEFs, as above. Notably, expression levels of
135 hcGAS-HA3x were similar to endogenous mouse cGAS (Figure 2B). As expected,
136 WT hcGAS-HA3x expression reduced VSV-GFP production, while expression of the
137 DNA binding and catalytically inactive hcGAS-HA3x mutants did not (Figure 2B).
138 These results indicate that cGAS-mediated restriction of an RNA virus depends on
139 its DNA binding and cGAMP synthase activities.

140 Because SV40 T antigen and other viral oncogenes can inhibit innate immune
141 responses, including cGAS-STING activation (37), we sought to confirm the above
142 findings in untransformed cells. We therefore reconstituted primary cGAS^{-/-} MEFs

143 with WT or mutant forms of hcGAS-HA3x and then assessed their ability to restrict
144 the growth of VSV-GFP, SINV-GFP, or VSV Δ M51A-GFP, a VSV mutant (M51A in
145 the M gene) that is more susceptible to innate immune responses (38). All three
146 viruses were significantly restricted in primary MEFs reconstituted with WT
147 hcGAS-HA3x but not with the DNA-binding nor cGAMP-synthase active site mutants
148 (Figure 3A-C). Restriction of VSV Δ M51A-GFP was more potent than VSV-GFP.
149 SINV-GFP was potently restricted by hcGAS WT but not by either DNA binding
150 mutant; SINV-GFP infection was modestly reduced in cells expressing the
151 E225A/D227A mutant.

152 To further corroborate the role of cGAS in restriction of RNA viruses in
153 immunocompetent human cells, we utilized the THP-1 human monocyte line that
154 has robust DNA sensing capability (21). First, we used CRISPR/Cas9 to generate
155 cGAS KO THP-1 monocytes, then established stable lines reconstituted with WT or
156 mutant hcGAS-HA3x; it should be noted that hcGAS-HA3x was overexpressed 2- to
157 6-fold in THP-1 cells relative to endogenous hcGAS (Figure S2). Differentiated WT
158 THP-1 cells and THP-1 KO cells reconstituted with WT hcGAS-HA3x restricted
159 growth of VSV-GFP, VSV Δ M51A-GFP, and SINV-GFP, while THP-1 KO cells or
160 THP-1 KO cells reconstituted with inactive hcGAS-HA3x mutants showed little or no
161 restriction (Figure 3D–3F). As observed previously in MEFs, VSV Δ M51A-GFP was
162 more potently restricted than VSV-GFP, but unlike in MEFs, infected fewer cells
163 expressing mutant cGAS. This was also true for SINV-GFP, albeit restriction with
164 WT hcGAS-HA3x was extremely potent, comparatively. It is unclear whether these
165 modest decreases in infection of the cGAS mutants was due to hcGAS-HA3x
166 overexpression in THP-1 cells, residual hcGAS activities, or normal clonal
167 variation of cells. Nevertheless, these results are most consistent with an integral
168 role for cGAS DNA binding and cGAMP synthase activities in RNA virus restriction.

169 **Detection of cGAMP produced in response to DNA transfection but not**
170 **RNA virus infection.** Because cGAMP synthesis activity was essential for RNA
171 virus restriction, we next sought to identify cGAMP produced in response to RNA

172 virus infection or, as a positive control, DNA transfection, by using liquid
173 chromatography-mass spectrophotometry (LC-MS) and LC-MS/MS. HEK 293E cells
174 were used in these experiments because this cell line lacks endogenous cGAS
175 expression and could be reconstituted with WT or mutant hcGAS-HA3x; however,
176 unlike MEFs and THP-1 KO cells, HEK 293E cells could be efficiently transfected
177 with DNA and readily scaled up for isolation of cGAMP from cytosolic extracts. As
178 shown in Figure 4A, a unique UHPLC peak (~5 minutes elution) was observed after
179 transfecting WT hcGAS-HA3x-expressing HEK 293E cells with salmon sperm DNA;
180 MS analysis confirmed that this peak corresponded to cGAMP (Figures 4B and 4C).
181 Moreover, cGAMP was not observed in untransfected cells expressing WT cGAS or
182 in DNA-transfected cells expressing a catalytically inactive form of hcGAS (Figure
183 4D). Surprisingly, cGAMP remained below detectable levels after 5 hours of
184 VSV-GFP infection at a MOI of 10 (Figure 4D), suggesting that detectable levels of
185 cGAMP were not produced in response to RNA virus infection.

186 While the LC/MS technique provides exquisite specificity for identifying cGAMP
187 in complex cytosolic extracts, cGAMP biological assays may be more sensitive.
188 Indeed, our UPLC-MS configuration reliably detected nanogram amounts of cGAMP
189 spiked into cytosolic extract (Fig. 4E), which equates to >1 million molecules of
190 cGAMP per cell. We therefore established a bioassay for cGAMP-mediated IRF-3
191 activation in streptolysin O- (SLO)-permeabilized cells (Figure 4F). This bioassay
192 was shown to be dependent on STING activation (Fig. 4G) and had a limit of
193 detection (L.O.D.) of $\sim 5 \times 10^{-4} \mu\text{g}/\mu\text{l}$ ($\sim 0.74 \mu\text{M}$) cGAMP (Figure 4H), in line with other
194 published cGAMP bioassays (21). Again, we were unable to detect cGAMP in
195 lysates from VSV-infected or SINV-infected THP-1 cells expressing WT hcGAS,
196 while a synthetic cGAMP control led to robust phosphorylation of IRF3 (Figure 4I).
197 To validate that cell-derived cGAMP could be detected by this assay, a time-course
198 experiment was conducted by transfecting HEK 293E cells expressing WT hcGAS
199 with salmon sperm DNA, revealing the time-dependent increase in cGAMP (Figure
200 4J). Furthermore, we found that transfected cGAMP was rapidly turned over within

201 hours (Fig. 4K), most likely via the ENPP1 phosphodiesterase previously reported to
202 turnover cGAMP in mammalian cells (39). Collectively, these results indicate that if
203 cGAMP is produced in response to RNA virus infection, it may be produced at levels
204 below the limit of our detection and/or rapidly turned over.

205 **cGAS binds mitochondrial DNA at steady state and during RNA virus**
206 **infection.** Given that cGAS DNA binding activity was also required for RNA virus
207 restriction, we sought to identify DNA ligands of cGAS during RNA virus infection.
208 First, we identified conditions to specifically co-immunoprecipitate cGAS and
209 mtDNA, a known DNA ligand (34). As shown in Figure 5A, mtDNA was specifically
210 enriched by HA-immunoprecipitation from cells expressing WT hcGAS-HA3x, but
211 not from cells expressing the K384E DNA binding mutant. It should be noted that this
212 experiment is representative of many iterations performed at different scales. Given
213 prior links between virus infection, mitochondrial stress, and cGAS-mtDNA
214 interaction (34, 40), we next asked whether VSV altered the amount of
215 cGAS-associated mtDNA. Surprisingly, VSV-GFP infection had no impact on the
216 amount of cGAS-associated mtDNA (Fig. 5B), which led us to isolate cytosolic DNA
217 (Figure 5C) to quantitate mtDNA content with and without infection. Unexpectedly,
218 VSV-GFP infection had no impact on either the total amount of cellular mtDNA (Fig.
219 5D) or cytosolic mtDNA (Fig. 5E).

220 To more broadly assess cytosolic and hcGAS-bound DNAs, we developed deep
221 sequencing libraries from cytosolic extracts or after immunoprecipitation of WT
222 hcGAS-HA3x. The first one-third of the mitochondrial genome was specifically
223 enriched in cytosolic preps from both uninfected and VSV-GFP-infected MEFs
224 (Figure 5F). Similarly, mtDNA was also highly enriched after immunoprecipitation of
225 hcGAS-HA3x, although there was a bias for the latter three-quarters of the genome
226 (Figure 5G). Importantly, there was no obvious difference in mtDNA pulldown
227 between uninfected and infected cells. Collectively, these data indicate that VSV
228 does not induce cytosolic release of mtDNA to stimulate cGAS activation. Consistent
229 with this, VSV-GFP replicated equally well in LMTK cells and mtDNA-depleted LMTK

230 ρ^0 cells (41), which express cGAS and STING (Figure 5H). Collectively, these data
231 suggest that mtDNA is dispensable for cGAS-mediated restriction of an RNA virus.

232 Although VSV is a negative-strand RNA virus that replicates solely via RNA
233 intermediates, it has been reported that VSV-specific cDNAs can arise in infected
234 cells, presumably through reverse transcriptase (RT) activity encoded by
235 endogenous retroelement(s) (42). We therefore investigated whether such viral
236 cDNAs arose during VSV-GFP infections in our laboratory. Indeed, VSV N
237 gene-specific cDNAs were generated in infected cells, although in extremely low
238 abundance, \sim 1 copy/ 10^4 cells (Figure 5I). The cDNA origin of the N gene template
239 was confirmed by nuclease treatment (Figure 5J), by its sensitivity to tenofovir, an
240 RT inhibitor that had no effect on VSV replication (Figure S3A), and by its enhanced
241 expression in cells devoid of TREX1 nuclease (Figure S3B). We also identified
242 virus-specific cDNAs in cells infected with yellow fever virus (YFV), a positive-strand
243 RNA virus (Figure S3C), suggesting that cDNA formation is a general feature of RNA
244 virus infections. Finally, to determine whether cDNA formation was specific to
245 virus-infected cells or to viral transcripts, we examined whether cDNA forms of an
246 abundant housekeeping gene, GAPDH, arose in uninfected cells. Indeed,
247 splice-dependent GAPDH cDNAs were identified in low abundance by qPCR (Figure
248 5K). Importantly, VSV or retroelement cDNAs were not detected in deep sequencing
249 analyses of whole cytosol or cGAS-HA immunoprecipitations, likely due to their low
250 abundance.

251 Collectively, our results indicate that cGAS binds mtDNA in both infected and
252 uninfected cells, and that VSV infection does not induce the release of mtDNA into
253 the cytosol or increase cGAS-bound mtDNA. Additionally, viral and cellular
254 mRNA-specific cDNAs can be detected, but are of extremely low abundance, less
255 than one copy per 10^4 cells. Taken together, these results suggest that steady state
256 levels of cytosolic DNA, rather than virus-induced DNAs, may provide ligands for
257 cGAS-mediated restriction of RNA virus replication.

258 **cGAS primes smoldering baseline ISG expression.** Based on the above
259 results, we hypothesized that cGAS may serve to program baseline levels of innate
260 immune activation rather than strictly in response to RNA virus infection. To address
261 this, we analyzed ISRE-driven luciferase expression in uninfected THP-1 cells
262 devoid of cGAS expression or reconstituted with WT or mutant hcGAS-HA3x (Figure
263 6A). These experiments suggested that WT hcGAS-HA3x significantly enhances
264 baseline ISG induction compared to the parental cGAS KO line and hcGAS mutants.
265 Further experiments showed that WT hcGAS-HA3x also stimulated greater
266 ISRE-driven luciferase production during infection of THP-1 cells with VSV-GFP and
267 SINV-GFP (Figures 6B, C). It should be noted that VSV-GFP ISG levels were not
268 appreciably different from the control, likely due to the transcriptional repression
269 capability of the M protein (43, 44).

270 To confirm our ISRE-luciferase findings, we used RT-qPCR to quantify ISG
271 transcripts known to be induced by the cGAS-STING DNA sensing pathway (Figures
272 6D–6G). These results show that cGAS KO significantly reduced basal expression of
273 Mx1 and CXCL10 in uninfected cells, but not of IFIT1, which was not expressed
274 basally. Importantly, cells reconstituted with WT hcGAS-HA3x expressed
275 significantly higher levels of IFIT1 and CXCL10 mRNA, while cells expressing
276 inactive hcGAS-3xHA mutants did not. As these experiments were conducted in
277 cells that slightly overexpressed hcGAS (Figure S2), ISG upregulation likely reflects
278 reinforced, native patterns of expression.

279 To examine whether cGAS drives basal levels of innate immune activation *in*
280 *vivo*, we examined ISG expression in vaginal tissue from uninfected WT B6J mice or
281 in mice defective for several innate immunity pathways. As shown in Fig. 7, low basal
282 levels of USP18, Mx1, and Rsad2 expression were observed in B6J mice, but were
283 significantly decreased in IFNAR1^{-/-} mice, demonstrating that basal ISG expression
284 depends on IFNAR signaling. Importantly, cGAS^{-/-} mice had significant decreases in
285 basal Mx1 and Rsad2 expression, similar in degree to reduced basal USP18 and

286 Rsad2 expression observed in IRF3/7^{-/-} mice. In contrast, MAVS had little effect on
287 basal ISG expression.

288 Altogether, these results suggest that cGAS primes cells to express smoldering
289 levels of ISG expression and that the DNA binding and catalytic activity are integral
290 to this phenomenon.

291

292 **Discussion**

293 While the RLR-MAVS and cGAS-STING pathways are important, respectively,
294 for restricting RNA and DNA virus infections, there is considerable crosstalk and
295 redundancy between these two pathways. For instance, mammalian RNA
296 polymerase III can transcribe A-T-rich DNA in the cytosol, producing uncapped
297 RNAs that trigger RIG-I (45, 46). In addition, STING can physically associate with
298 RIG-I and MAVS and may act as a cofactor in RNA sensing (47-49). More recently,
299 STING has been shown to inhibit RNA virus replication, independent of ISG
300 expression, via translational control (20).

301 Although cGAS was previously reported to restrict RNA viruses (50), it has been
302 widely assumed — though unproven — that this restriction depends on cGAS's DNA
303 binding and cGAMP synthase activities. Here, we used genetic knockout and
304 transgenic replacement to determine that both DNA binding and cGAMP synthase
305 activities are essential for cGAS-mediated restriction of RNA viruses. One caveat to
306 this approach is that gene knockout can have far-reaching network-level effects on
307 transcription, which are just beginning to be unearthed (51). A second caveat is that
308 reconstituted cGAS was slightly overexpressed in THP-1 cells, which, at least for WT
309 cGAS, can induce ISG expression (50, 52) and may have exaggerated the
310 response. Nevertheless, our results in THP-1 cells were consistent with results
311 obtained from MEFs (Figure 3), which did not overexpress cGAS. Taken together,
312 these data establish that DNA binding and cGAMP synthase activities are required
313 for cGAS-mediated RNA virus restriction.

314 Despite the essential role of cGAMP synthase activity and demonstrated
315 detection of cGAMP synthesized after DNA transfection, we were unable to detect
316 cGAMP production in response to VSV-GFP infection. Our results are consistent
317 with results recently reported by Franz et al., who were also unsuccessful in
318 detecting cGAMP production in VSV-infected cells (20). While Franz and colleagues
319 concluded that cGAMP is not produced in response to VSV infection, we also
320 considered the possibility that cGAMP levels may be below the limit of detection
321 and/or rapidly degraded. Whereas cGAMP synthesis is readily detected in response
322 to DNA transfection, this may simply reflect the wide dynamic range of cGAS in
323 response to overloading the cytosol with transfected DNA. Moreover, it has been
324 exceedingly difficult to detect cGAMP after virus infections, even for DNA viruses.
325 For instance, Paijo et al. reported that the detection of cGAMP produced in response
326 to cytomegalovirus infection was cell type-dependent, despite active cGAS-STING
327 expression. Where cGAMP was detected, levels were on the order of 5 fmol/10⁴
328 cells, or ~3x10⁵ molecules/cell, which was slightly above their assay's limit of
329 detection (53). As our biochemical and biological assays were both less sensitive
330 than that of Paijo et al., we surmise that the synthesis of cGAMP in response to RNA
331 virus infection is below the limit of detection and/or may be rapidly turned over.
332 Alternatively, continuous low-level production of cGAMP in response to endogenous
333 DNA ligands may be more relevant to RNA virus restriction. Clearly, cGAMP assays
334 with improved sensitivity are needed to discern between these possibilities.

335 Because cGAS DNA binding activity was required for VSV restriction, we
336 examined whether VSV introduces cGAS DNA ligands into the cytosol. Prior work
337 has shown that the cytosolic release of mtDNA activates the cGAS-STING pathway
338 (33-35); moreover, infection with HSV-1, a DNA virus, or dengue virus, an RNA
339 virus, reportedly causes cytosolic release of mtDNA (34, 40). An emerging concept
340 is that mammalian cells may regulate the efflux of mtDNA into the cytosol in
341 response to stress, supported by a role for the Bax/Bak pore in mtDNA release as
342 well as mitochondrial inner membrane release mechanisms via permeabilization and

343 herniation (33-35, 54, 55). In contrast, the levels of cytosolic mtDNA and
344 cGAS-associated mtDNA did not increase during VSV infection. Moreover,
345 cGAS-STING-mediated VSV restriction was intact in ρ^0 cells, which lack mtDNA,
346 consistent with similar experiments reported by Franz et al. (20). Real-time
347 examination of mitochondrial dynamics may be needed to clarify the role of mtDNA
348 release during RNA virus infections.

349 Given that cGAS recognizes RNA:DNA hybrids(22), as well as a recent report of
350 VSV cDNAs (42), we also quantitated viral cDNAs produced during VSV infection.
351 We confirmed that rare viral and cellular cDNAs are indeed produced, most likely by
352 an endogenous cellular RT; however, the abundance of any given cDNA was
353 incredibly low, ~1 copy per 10^4 cells. This was less than the amount of VSV N-gene
354 cDNA previous reported by Shimizu, et al. (42), which we attribute to the enhanced
355 specificity of our hydrolysis probe-based assay vs. SYBR green assays.
356 Nevertheless, the low abundance of VSV cDNAs is inconsistent with a model
357 whereby RNA virus cDNAs play a significant role in stimulating population-wide
358 innate immune responses. These findings, however, do highlight the constant
359 synthesis and turnover of cDNAs within the mammalian cytosol. Consistent with this,
360 deficiencies in the TREX1 nuclease lead to cytosolic accumulation of DNA, including
361 retroelement cDNAs, causing chronic cGAS stimulation and autoimmunity in the
362 form of Aicardi-Goutières syndrome (4, 56, 57).

363 Given that cGAS may be continuously stimulated by endogenous DNA ligands,
364 and that candidate DNA ligands were unchanged during VSV infection, we
365 examined whether cGAS contributes to a pre-existing baseline of innate immune
366 activation. Indeed, low level cGAS-dependent ISG expression was observed even in
367 the absence of viral infection and was significantly decreased in cGAS KO cells,
368 consistent with prior examples of the cGAS-STING pathway altering ISG baseline
369 expression (2, 50, 58, 59). These results support the hypothesis that cGAS
370 contributes to RNA virus restriction by establishing smoldering, baseline-levels of
371 constitutive innate immune activation. This is an important distinction from other

372 models where cGAS responds to RNA virus-induced release of mtDNA. Additional
373 work will be needed to definitively identify the relevant DNA ligands that activate
374 cGAS; we suggest that pre-existing baseline stimuli should be considered.

375 While ISG expression served as a convenient and sensitive readout of baseline
376 cGAS-STING activation in our studies, it should be noted that we did not
377 demonstrate that low-level ISG expression directly contributes to RNA virus
378 restriction. On the surface, our results may seem at odds with those of Franz et al.,
379 who recently reported that STING restricts RNA viruses, including VSV, in an
380 ISG-independent manner (20). However, we do not exclude the possibility that
381 smoldering cGAS activation may also contribute to ISG-independent mechanisms of
382 virus restriction via STING.

383 In summary, we propose that cGAS may become activated in response to RNA
384 virus infection, such as by virus-induced mtDNA release, but also contributes to RNA
385 virus restriction via constitutive, low-level innate immune activation, likely via
386 recognition of endogenous DNA ligands (Fig. 8).

387 **Materials and Methods**

388 **Animal research.** All mice were maintained, bred, and handled in our facility in
389 compliance with federal and institutional policies under protocols approved by the
390 Yale Animal Care and Use Committee. C57BL/6J, B6(C)-Cgas^{tm1d(EUCOMM)Hmgu/J}
391 (*cGAS*^{-/-}) mice (50) and B6(Cg)-Ifnar1tm1.2Ees/J (*Ifnar1*^{-/-}) mice (60) were
392 purchased from Jackson Laboratory. *Irf3*^{-/-} *Irf7*^{-/-} mice (61) were a generous gift by
393 Dr. T. Taniguchi (University of Tokyo) and *Mavs*^{-/-} mice (62) were a generous gift by
394 Dr. Z. Chen (University of Texas, Southwestern).

395 Primary MEFs were isolated from day 14.5 embryos (E14.5) as previously
396 described (63). Vaginal tissue was harvested from six- to twelve-week old female
397 mice synchronized in diestrus via subcutaneous injection with 2 mg Depo-Provera in
398 the neck scruff; mice were sacrificed and vaginal tissue harvested ten days after
399 Depo-Provera treatment.

400 **Cell cultures.** All cells were maintained at low passage by using a seed-lot
401 system and routinely tested for mycoplasma contamination. HEK 293E cells were
402 obtained from Dr. W. Mothes (Yale); SW13 cells were obtained from Dr. C. Rice
403 (Rockefeller University); BHK-21 cells were obtained from Dr. D. Brackney (State of
404 Connecticut Agriculture Research Station); LMTK and LMTK ρ^0 cells were obtained
405 from Dr. G. Shadel (Yale). HEK293E, SW13, BHK-21 and MEF cells were
406 maintained at 37°C and 5% CO₂ in complete growth medium (DMEM containing 2
407 mM L-glutamine [Invitrogen], 10% heat-inactivated fetal calf serum [FCS, Omega
408 Scientific], and 0.1 mM non-essential amino acids [NEAA; Invitrogen]). LMTK and
409 LMTK ρ^0 cells were maintained in DMEM as above supplemented with 100 μ g/mL
410 sodium pyruvate (Invitrogen) and 50 μ g/mL uridine (Sigma)

411 THP-1-Lucia ISG cells (Invivogen) were maintained at 37°C and 5% CO₂ in
412 RPMI 1640 containing 2 mM L-glutamine, 10% FCS, 0.1 mM NEAA, 10 U/mL
413 penicillin/streptomycin, 100 μ g/mL normocin, and 100 μ g/mL zeocin.

414 Pilot experiments showed that THP-1-derived macrophages were more
415 permissive for SINV-GFP than undifferentiated THP-1 monocytes. Differentiation of

416 THP-1 monocytes to macrophages was performed by plating cells at a concentration
417 of 5×10^5 cells/mL in fresh media and incubating for three days with 100 ng/mL
418 phorbol myristate acetate (PMA; Invivogen). Adherent monolayers were washed
419 once with DPBS, dissociated with 0.05% trypsin/EDTA, resuspended in fresh media,
420 counted, and seeded for experimentation.

421 **Viruses.** Viruses expressing green fluorescent protein (GFP) were used to
422 facilitate monitoring of virus infections. rVSV-p1-eGFP (VSV-GFP) (64) and
423 rVSV-ΔM51-p5-eGFP (VSVΔM51A-GFP) (65) were kind gifts from Drs. J. Rose and
424 A. van den Pol (Yale), respectively. SINV G100-eGFP (SINV-GFP) (66) was a kind
425 gift from Dr. M. Heise (University of North Carolina, Chapel Hill). VSV and SINV
426 stocks were generated via low multiplicity of infection (MOI = 0.01) passage in
427 BHK-21 cells. Herpes simplex virus 1 KOS-eGFP (HSV-GFP) (67), kindly provided
428 by Dr. P. Desai (Johns Hopkins Medical School), was propagated by low MOI
429 passage (MOI 0.01) in Vero cells, and harvested at 60 hours post-infection.
430 HSV-GFP was prepped by three cycles of freezing (-80°C) and thawing (37°C),
431 clarification (1,500 x g for 15 minutes at 4°C), addition of 10% FCS and 7% dimethyl
432 sulfoxide, aliquoted, and stored at -80°C.

433 **Plaque assay and fluorescent cell counting.** Plaque assays were developed
434 by using semi-solid overlays (DMEM, 10% FCS, 1.6% LE agarose). When plaque
435 formation was evident, cells were fixed with 3% formaldehyde, agarose plugs were
436 removed, and cells stained with 0.1% crystal violet in 20% ethanol. Plaque forming
437 units per mL (pfu/mL) were calculated by counting the number of colonies formed
438 and multiplying this count by the dilution factor.

439 To prepare GFP-expressing cells for cytometry, cells were trypsinized, washed
440 with DPBS, and fixed in DPBS containing 1% PFA. Fluorescent cells were counted
441 on an Accuri C6 Flow Cytometer (Becton-Dickinson).

442 **Protein analysis.** For western blotting, cells were lysed in RIPA buffer (50 mM
443 Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS)
444 containing protease inhibitor cocktail, followed by a 20-minute spin at 16,100 x g and

445 4°C to remove insoluble material. Protein concentrations were quantified by using a
446 BCA protein assay kit (Thermo Scientific). Equal amounts of protein were separated
447 on 4-12% Bis-Tris Bolt SDS-PAGE gels (Thermo Scientific) and transferred to PVDF
448 membranes by using a Pierce Fast Semi-Dry Blotter. Immunoblotting was performed
449 by 30 minutes of blocking with either 5% milk (American Bio) or SuperBlock (Thermo
450 Scientific) followed by primary antibody and then secondary antibody (2 hours and 1
451 hour at room temperature, respectively), diluted in the same blocking solution. Blots
452 were developed by using SuperSignal Pico or Femto chemiluminescence substrate
453 kits (Thermo Scientific) and imaged on a GE ImageQuant LAS 4000. Precision Plus
454 protein standards (Bio-Rad) were used to estimate protein molecular weights.

455 The following primary antibodies were used for western blotting analysis: Rabbit
456 anti-HA (1:1,000, Abcam #ab9110), rabbit anti-pIRF3 (1:1,000, Abcam #76493),
457 rabbit anti-cGAS (1:500, CST #15102s), rabbit anti-TOM40 (1:5,000, Santa Cruz
458 #H-300), rabbit anti-Calreticulin (1:5,000, Abcam #ab2907), rabbit anti-Lamin B1
459 (1:1,000, Abcam #ab16048), and mouse anti-β-actin (1:10,000, Sigma #A1978).
460 The following secondary antibodies were used for western blotting analysis: Goat
461 anti-rabbit horseradish peroxidase (1:5,000, Jackson ImmunoResearch
462 #111-035-144), and goat anti-mouse horseradish peroxidase (1:5,000, Jackson
463 #115-035-146).

464 To immunoprecipitate cGAS-DNA complexes, hcGAS-HA3x-expressing cells
465 were fixed in DPBS containing 0.5% paraformaldehyde (5 minutes, room
466 temperature), then quenched with 125 mM glycine. All subsequent steps were
467 performed at 4°C. After two washes with DPBS, cells were lysed for 30 minutes in
468 ice-cold RIPA, followed by a 20-minute spin at 16,100 x g. Clarified lysates were
469 sonicated with four cycles of 10 seconds on and 30 seconds off at 20% amplitude on
470 a Sonifier 450 (Branson Ultrasonics). Samples were spun for 20 minutes at 16,100 x
471 g and supernatants were retained.

472 To perform immunoprecipitation, lysates were pre-cleared with 2 µg/mL rabbit
473 sera and two incubations with 50 µL protein A-magnetic beads (Pierce). Samples

474 were then rotated overnight with 2 µg HA antibody, and complexes were captured
475 with Protein A-magnetic beads. Washing was performed as follows: 2x with RIPA, 2x
476 with high salt RIPA (500 mM NaCl), 1x with IP-wash buffer (0.5 M LiCl, 1% NP-40,
477 1% deoxycholate, 100 mM Tris-HCl pH 8.0), and 2x with T₁₀E₁ (10 mM Tris-HCl pH
478 8.0, 1 mM EDTA). Bound complexes were eluted with 0.1 M glycine-HCl, pH 2.5,
479 samples were neutralized with 1 M Tris-HCl pH 8.0 (0.1 M final), and eluted
480 protein-nucleic acid complexes were then processed for western blotting or deep
481 sequencing (see Nucleic acid purification, below).

482 **PCR, qPCR, and RT-PCR.** Standard PCRs were performed with Phusion DNA
483 polymerase or Taq DNA polymerase (NEB). Unless otherwise noted, cycling was
484 performed for 35 cycles with primers listed in Table 1.

485 For RT-PCR, RNAs were extracted from cells by using TRIzol Reagent (Life
486 Technologies) or the RNeasy extraction kit (Qiagen). Viral RNA was extracted from
487 cell culture media with the QiAmp Viral RNA Mini Kit (Qiagen). cDNA synthesis was
488 performed by using random hexamer or gene-specific primers with the Transcriptor
489 First Strand cDNA Synthesis Kit (Roche).

490 For qPCR of cell culture-derived cDNAs, primers were designed by using the
491 ProbeFinder software (Roche) for compatibility with Roche Universal Probe Library
492 (UPL) hydrolysis probes. Assays were performed in a LightCycler 96 or LightCycler
493 480 (Roche), as per manufacturer's instructions, with primers and UPL probes listed
494 in Table 1. All reactions were performed in duplicate and quantified by comparison to
495 standard curves created with cloned amplicons diluted (10² – 10⁷ copies) in ddH₂O
496 supplemented with 50 ng/µL carrier DNA and run in parallel.

497 For RT-qPCR of mouse tissue-derived mRNAs, SYBR Green qPCR reactions
498 were run in triplicate with gene specific primers (Table 1). The Ct values were
499 averaged, internally normalized against housekeeping gene HPRT, then normalized
500 to B6J control mice by using the $\Delta\Delta Ct$ method of comparison. Fold-expression was
501 estimated assuming one doubling per cycle (fold expression = 2^{- $\Delta\Delta Ct$}).

502 **Plasmids, lentiviruses, and retroviruses.** pMXs-hcGAS-HA3x-IRES-puro
503 was made by PCR amplifying hcGAS-HA3x from pUNO1-hcGAS-HA3x (Invivogen)
504 with YO-2142 and YO-2143 and cloning into pMXs-mcGAS-HA3x-IRES-puro (gift of
505 Dr. G. Shadel, Yale University) via common Xhol and NotI sites. To facilitate
506 reconstitution of puromycin-resistant cGAS knockout (KO) cells with hcGAS, the Pac
507 gene in pMXs-hcGAS-HA3x-IRES-puro was replaced with the Bsd gene, amplified
508 from pMICU-APEX2 (68) (Addgene plasmid # 79057). In addition, the 518-bp NotI–
509 BpI fragment of hcGAS was recoded (BlueHeron) to avoid editing of the
510 reconstituted hcGAS. For transient expression of hcGAS in primary MEFs,
511 hcGAS-3xHA was cloned into pLenti-puro (69) (Addgene plasmid # 39481).

512 Site-directed mutagenesis of hcGAS was performed by using appropriate
513 primers (Table 1) and PfuTurbo (Agilent Technologies), as previously described
514 (70). Mutants were sequenced and subcloned back into the pMXs-IRES-puro vector
515 with Xhol and NotI.

516 Gene knockout was performed in cell culture by using Cas9 to induce
517 non-homologous end-joining repair. Briefly, gRNAs-specific oligos (Table 1) were
518 chosen from published datasets (71) or designed with gRNA Designer (72) and
519 cloned into pLentiCRISPR (73) (Addgene plasmid # 51760).

520 **Lentiviruses and retroviruses.** Lentiviruses and retroviruses were packaged in
521 HEK 293E cells by co-transfection with appropriate HIV- or MLV-Gag/Pol and VSV G
522 packaging constructs. Forty-eight hours post-transfection, packaged vector stocks
523 were clarified (16,100 x g), passed through a 0.45 µm filter, and supplemented with 8
524 µg/mL polybrene (Sigma) and 20 mM HEPES (Life Technologies). Target cells were
525 transduced by spinoculation, selected with 3 µg/mL puromycin, and screened for
526 expression or knockout via genomic PCR and sequencing and/or western blotting.
527 To develop clonal cultures, adherent cells were isolated by using sterile 8 mm Pyrex
528 cloning cylinders and expanded. Clonal phenotypes were screened via western
529 blotting or qPCR.

530 **Luciferase assays.** Lucia luciferase-containing samples were clarified by
531 centrifugation (16,100 \times g for 5 minutes) and mixed with 1/4-volume of 5x Renilla lysis
532 buffer (Promega) to destroy virus infectivity. Lucia activity in 20 μ L samples was
533 measured on a Centro LB 960 plate reader (Berthold) by integrating over 10
534 seconds.

535 **cGAMP extraction and assays.** Cells were infected for five hours with
536 VSV-GFP or SINV-GFP (MOI 3–10), transfected for five hours with salmon sperm
537 DNA (2 μ g/mL final concentration) and Transit LT-1 (Mirus), or left untreated.
538 cGAMP was extracted based on established methods (21). Briefly, cells were
539 dissociated with trypsin, washed with DPBS, gently pelleted and resuspended at a
540 concentration of 1×10^7 cells/mL in ice-cold cGAMP homogenization buffer (10 mM
541 Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂). Cells were lysed via nitrogen
542 cavitation in a cell disruptor (Parr Instrument Company). Lysates were clarified at
543 1,000 \times g for 5 minutes, then 16,100 \times g for 10 minutes, retaining the supernatants.
544 Resulting supernatants were digested for 1 hour with benzonase (0.5 U/ μ L; Fisher
545 Scientific) at 37°C, 1 hour with proteinase K (0.5 U/ μ L; Invitrogen) at 55°C,
546 heat-inactivated at 95°C for 10 min, and spun for 5 minutes at 16,100 \times g, retaining
547 the final supernatant (S1).

548 To detect cGAMP bioactivity, 2 μ L of S1 sample, synthetic cGAMP (positive
549 controls), or DPBS (negative controls) were incubated with 10^6 THP-1 cells, 2 mM
550 ATP, 1.5 ng/ μ L SLO (a kind gift from Dr. N. Andrews, University of Maryland) and
551 media in 8 μ L (total volume). After 1.5 hours at 30°C, reactions were lysed with an
552 equal volume of RIPA buffer and processed for phosphorylated IRF3 western blot,
553 as above.

554 To detect cGAMP by liquid chromatography and mass spectrophotometry,
555 trypsinized cells were washed once with DPBS, pelleted at 1,000 \times g and then frozen
556 at -20°C. To extract cGAMP, 5×10^6 cells/mL were resuspended three times in
557 extraction buffer (40% acetonitrile, 40% methanol, 20% ddH₂O) for 20 minutes,
558 spinning after each extraction at 16,100 \times g and keeping the supernatant.

559 Supernatants were pooled, dried overnight in a GeneVac HT-8 (SP Scientific), and
560 resuspended in 100 μ L ddH₂O per 5x10⁶ cells. Samples were filtered with a 0.2 μ m
561 PTFE syringe filter (VWR) prior to loading into a Luna Omega C18 UHPLC column
562 (Phenomenex) on an iFunnel 6550 Q-TOF / MS (Agilent). Samples were run in
563 negative mode with the following parameters: Buffer A = 0.1% formic acid; Buffer B =
564 acetonitrile, 0.1% formic acid; gradient cycles: 0 – 4% B over 10 minutes, 4% B –
565 100% B over 5 minutes, 5 minutes wash with 100% B; UV detection at 260 nm, m/z
566 scans from 150-1,000. cGAMP was observed between 4–7.5 minutes in extracted
567 ion chromatographs at an observed mass of 673.085 m/z; this was confirmed to be
568 cGAMP by MS/MS ion fragmentation patterns.

569 **Preparation of cytosolic nucleic acid extracts.** Cells were trypsinized and
570 resuspended in an equal volume of fresh media, then spun at 1,000 x g for 5 minutes
571 at room temperature. After washing once with DPBS, cells were resuspended in
572 cytosolic extraction buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 25 μ g/mL digitonin)
573 and incubated for 10 minutes at 4°C with rotation. A succession of 4°C spins was
574 performed, retaining the supernatant for each step: 3x 1,000 x g for 3 minutes, 1x
575 16,100 x g for 10 minutes, 1x 100,000 x g for 1 hour on a 0.34 M sucrose cushion
576 (SW41 Ti rotor, Beckman). The final supernatant was then processed for western
577 blotting, above, and DNA purification, below.

578 **DNA purification and phi29 amplification.** DNA was isolated from total cytosol
579 by treating samples with RNase A and RNase T1 (Ambion) for 1 hour at 37°C,
580 digesting with Proteinase K for 1 hour at 55°C, and heat inactivating at 95°C for 15
581 minutes. DNAs were then purified with the QiaQuick purification kit (Qiagen). To
582 isolate DNA from immunoprecipitates, crosslinks were reversed by adding 5 M NaCl
583 (0.3 M final) and shaking overnight at 65°C, then digesting RNA and protein, as
584 above. For isothermal DNA amplification, 1–40 ng of DNA was annealed to
585 exo-resistant random hexamer primers (Molecular Cloning Laboratories) and
586 amplified overnight at 30°C with phi29 DNA polymerase (NEB), followed by a 65°C
587 inactivation step. DNA was extracted with the QiaQuick purification kit.

588 **Sequencing library preparation, sequencing, and analysis.** Phi29-amplified
589 samples were sonicated, as above, to achieve DNA fragments of 200- to 500-bp.
590 DNAs were end-repaired with T4 DNA polymerase (NEB), T4 polynucleotide kinase
591 (NEB), and Klenow DNA polymerase (NEB) at 20°C for 30 minutes, then purified via
592 QiaQuick. A-tailing was performed with Klenow fragment (3'-5' exo[-], NEB) before
593 ligation of TruSeq Adapters (Illumina) and amplification with Phusion DNA
594 Polymerase (NEB) and Illumina TruSeq primer cocktails. Size-selected libraries
595 (350- to 450-bp) were excised from LMP agarose, purified with a Gel Extraction Kit
596 (Qiagen), re-amplified by PCR for 18 cycles, then purified. Triplicate libraries were
597 sequenced on a HiSeq4000 (Illumina) by the Yale Center for Genome Analysis with
598 a 150-bp paired-end protocol, 100 million reads/sample.

599 **Software and Statistics.** Unless otherwise noted, statistical significance was
600 estimated by using the Student's t-test with Holm-Sidak correction for multiple
601 comparisons. Standard p-value indicators were used throughout the manuscript: *
602 indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001; and **** indicates
603 p<0.0001. Data were graphed by using Graphpad Prism software (version 7.0a).
604 Pixel densities were analyzed in ImageJ2 (74) and images were prepared for
605 presentation with Photoshop and Illustrator CS4 (Adobe). Next-generation
606 sequencing results were mapped to the mm10 mouse genome by using the
607 Burrows-Wheeler aligner (BWA) (75) and TopHat (76) to look for raw and gapped
608 alignment, respectively. Alignments were assessed for content of genomic DNA and
609 mtDNA with the integrated genome browser software (77).
610

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896

897

898 **Figure Legends**

899 **Figure 1. WT cGAS and hcGAS restrict RNA virus infection in immortalized**
900 **MEFs.** Single-step growth curve of (A) VSV-GFP and (B) SINV-GFP production from
901 MEFs infected at MOI 3, as assessed by plaque assay on SW13 cells. (C) VSV-GFP
902 stocks were titered by plaque assay on WT, KO, and KO+WT cells.

903

904 **Figure 2. hcGAS-HA3x variants display differential restriction of RNA virus**
905 **infection.** (A) The structure of hcGAS (grey) in complex with ATP (pink) and DNA
906 (yellow-purple), as rendered from PDB 6CTA; residues K384 (blue), K407 (green),
907 and E225/D227 (orange) are shown. (B) Plaque assay of VSV-GFP produced by
908 SV40 T-immortalized MEF KO cells reconstituted with hcGAS-HA3x variants and
909 infected (MOI 3) for 8 hours; western blot of hcGAS expression is shown below.

910

911 **Figure 3. hcGAS variants display differential restriction of RNA virus infection.**
912 Primary KO MEFs were transduced to express hcGAS variants with lentiviral vectors
913 and infected with (A) VSV-GFP, (B) VSVΔM51A-GFP, or (C) SINV-GFP; % infected
914 cells was determined by flow cytometry in relation to empty vector-transduced KO
915 MEFs (Empty). WT THP-1, THP-1 cGAS knockout (KO), and THP-1 cGAS KO cells
916 reconstituted with WT (+WT) mutant forms of hcGAS (K384E, K407E, or E/D) were
917 differentiated with PMA and infected with (D) VSV-GFP, (E) VSVΔM51A-GFP, or (F)
918 SINV-GFP; % infected cells was determined by flow cytometry in relation to THP-1
919 KO cells.

920

921 **Figure 4. VSV-GFP infection does not induce detectable cGAMP production.**
922 (A) UHPLC profiles showing a time-course of cGAMP production after transfecting
923 salmon sperm DNA into hcGAS-3xHA-expressing HEK 293E cells. The yellow box
924 represents the peak elution range of synthetic cGAMP observed in pilot
925 experiments. (B) Mass chromatogram of the eluted cGAMP peak after transfecting
926 DNA hcGAS-3xHA-expressing HEK 293E cells. Known ionization products of

927 cGAMP are highlighted in orange. (C) Diagram of cGAMP indicating predicted
928 fragmentation pattern from MS data of cell-derived cGAMP; for reference a mass
929 chromatogram obtained from synthetic cGAMP (Invivogen) is shown. (D) UHPLC
930 profiles of untreated, VSV infected, or DNA transfected HEK293E cells expressing
931 WT or catalytically inactive hcGAMP-3xHA. (E) Standard curve of extracted ion
932 currents vs. synthetic cGAMP input. (F) Workflow of the cGAMP bioassay, see text
933 for details. (G) cGAMP-mediated IRF3 phosphorylation is dependent on STING.
934 WT or STING KO THP-1 were transfected with cGAMP, DNA, or left untransfected;
935 TF Controls received transfection reagent but no DNA. pIRF3, STING, and β -actin
936 were detected by western blot. (H) Standard curve of pIRF3 detection vs. synthetic
937 cGAMP input; L.O.D., limit of detection. (I) cGAMP was not detected during RNA
938 virus infections. WT THP-1 or cGAS KO cells expressing the indicated forms of
939 hcGAS-HA3x were infected with VSV-GFP or SINV-GFP at MOI 3 for 5 hours. Data
940 are representative of multiple experiments performed at various scales and lengths
941 of infection. (J) Time-course of cGAMP formation after transfecting DNA into HEK
942 293E cells expressing hcGAS-HA3x. (K) Time-course of cGAMP activity in whole
943 cell lysates of HEK 293E cells transfected with cGAMP.

944

945 **Figure 5. VSV infection does not introduce cGAS DNA ligands.** (A) Isolation of
946 cGAS-bound mtDNA. The amount of mtDNA D-loop sequence was quantitated by
947 qPCR after HA-immunoprecipitation from MEF cGAS KO cells reconstituted with WT
948 hcGAS-HA3x or the K384E DNA binding mutant. The No Ab control was from WT
949 cells. This experiment was repeated many times at different scales, with similar
950 cGAS-specific enrichment of mtDNA. (B) The mtDNA content of VSV-GFP-infected
951 and uninfected MEFs was assessed by D-loop qPCR. (C) Western blotting of
952 organelle/compartment-specific proteins in MEF WT total and cytosolic fractions with
953 25 μ g/mL digitonin extraction. (D) Total amounts of mtDNA (Dloop and CytB) and
954 cellular DNA (β -gluc) were determined by qPCR in uninfected and
955 VSV-GFP-infected MEF cells. (E) The mtDNA content was determined in cytosolic

956 extracts from uninfected and VSV-GFP-infected MEF cells. (F) Deep sequencing of
957 cytosolic extracts from uninfected and VSV-GFP-infected MEFs revealed the
958 presence of mtDNA. (G) Deep sequencing of cGAS-immunoprecipitates from
959 uninfected and VSV-GFP-infected reveal abundant mtDNA. (H) Time course of
960 VSV-GFP infection in LMTK and LMTK ρ^0 cells; cGAS and STING expression were
961 confirmed by western blot (inset). (I) Detection of VSV cDNA in virus-infected cells. N
962 gene-specific primers and probes were used to quantitate VSV cDNAs. GAPDH was
963 used as a control for cellular target DNA. (J) VSV cDNAs are sensitive to DNase I.
964 Cytosolic extracts were incubated with the indicated nucleases, cleaned up, and
965 subjected to qPCR. (K) Detection of GAPDH cDNA. Total cellular DNA was
966 subjected to qPCR with genomic DNA (gDNA)- and splice dependent
967 (cDNA)-specific primer and probe sets.

968

969 **Figure 6. cGAS primes basal ISG expression in steady state cell cultures.**

970 ISRE-driven luciferase production in (A) uninfected, (B) VSV-GFP infected, and (C)
971 and SINV-GFP infected THP-1 KO cells lines with or without WT or mutant
972 hcGAS-3xHA expression. RT-qPCR of (D) MX1, (E) IFIT1, and (F) CXCL10
973 expression in uninfected WT THP-1, THP-1 KO, or THP-1 KO cells expressing WT
974 or mutant hcGAS-HA3x.

975

976 **Figure 7. cGAS primes basal ISG expression *in vivo*.** Vaginal tissue was
977 collected from uninfected female mice of the indicated genotypes, synchronized in
978 diestrus. Expression levels of USP18, Mx1, and Rsad2 were quantitated by
979 RT-qPCR and normalized to B6J mice.

980

981 **Figure 8. Model of cGAS-mediated restriction of RNA virus infection.**

982

983 **Figure S1. hcGAS recoding, gRNA binding site, and residues targeted for**
984 **mutations.** Site-directed mutagenesis was utilized to generate three mutants

985 (brown). The K384E and K407E mutations disrupt the DNA-binding ability of the
986 cGAS, while the E225A/D227A mutation ablates cGAMP catalytic activity. The 5'
987 518-bp of hcGAS were codon optimized (red) to improve expression and to generate
988 a sequence resistant to CRISPR/Cas9 targeting. The gRNA binding site (blue) was
989 modified at 8 residues.

990

991 **Figure S2. hcGAS KO in THP-1 cells and reconstitution with hcGAS-HA3x.**

992 Western blotting of (A) a dilution curve of THP-1 WT lysate inputs and (B) lysates of
993 THP-1 WT and hcGAS KO cells reconstituted with hcGAS. (C) Standard curve
994 generated from a dilution series of the lysate used in (A). (D) Calculation of relative
995 hcGAS expression levels in (B) as compared to the THP-1 WT sample by using the
996 standard curve in (C).

997

998 **Fig. S3. Detection of viral cDNAs.** (A) Time-course of VSV growth and cDNA
999 formation in HEK-293T cells treated with tenofovir and infected with VSV-GFP (MOI
1000 3). Tenofovir (1 μ M), which was added one hour prior to infection and maintained
1001 throughout the time-course, had no effect on VSV replication. (B) Accumulation of
1002 VSV N-gene cDNA in WT HEK 293 cells or HEK 293E cells ablated for TREX1 by
1003 CRISPR/Cas9. This experiment was repeated once with similar results. (C)
1004 Detection of YFV cDNA. Shown here is the standard curve used to estimate absolute
1005 DNA copies via qPCR (left) and the quantity of viral cDNA detected in BHK-21 or
1006 Huh-7.5 cells infected with YFV-17D (right). This experiment was repeated twice
1007 with similar results.

1009 **Table 1. Oligonucleotide primers and probes used in these studies**

Name	Use*	Sequence (5'- to -3')	U^
YO-1485	hIFIT1 F	agaacggctgcctaattacag	9
YO-1486	hIFIT1 R	gctccagactatccctgacctg	9
YO-1659	hSTING gRNA F	caccgggtctgctgagtcgcctgc	
YO-1660	hSTING gRNA R	aaacggcaggcactcagcagaaccc	
YO-1665	hTREX1 gRNA F	caccggcagtgggtgtacagcaga	
YO-1666	hTREX1 gRNA R	aaactctgcgtcacaaccactgcc	
YO-1925	YFV NS1 F	ggtaagaacccctgtgttctcc	69
YO-1926	YFV NS1 R	ggcattttcctggactttc	69
YO-1936	VSV N F	tgacaacacagtcgttagttcca	4
YO-1937	VSV N R	aatctgcgggtattccact	4
YO-2017	hD-loop F	ctcagatagggtcccttga	88
YO-2018	hD-loop R	gcactctgtgcgggatatt	88
YO-2037	hGAPDH F	ccccggttctataaattgagc	63
YO-2038	hGAPDH R	tttctctccgcggctt	63
YO-2039	hGAPDH cDNA F	ctctctgcctctgttcg	60
YO-2040	hGAPDH cDNA R	accaaattccgttactccga	60
YO-2041	h β Gluc F	tgtgtctgcagtgggtgaat	77
YO-2043	h β Gluc R	ggtattggatggccctgg	77
YO-2115	mGAPDH F	cagttgtccaaatttgttctagg	77
YO-2116	mGAPDH R	ttactccctggaggccatgt	77
YO-2119	mD-loop F	catcaacataggcgtcaagg	56
YO-2120	mD-loop R	tgggtttgcggactaatg	56
YO-2142	hcGAS-HA F	taagcactcgagatgcagcctggcacggaaag	
YO-2143	hcGAS-HA R	tgcttagccgcgttgcacatgtctggcacatc	
YO-2144	K384E F	gctatcctcttcacatcgaagaggaaatttgaacaatcatgg	
YO-2145	K384E R	ccatgattgtcaaaattcttcgtatgtgagagaaggatagc	
YO-2146	K407E F	gaaaacaaagaagagaaaatgtgcagggaaagattttaaaactaatgaaatacc	
YO-2147	K407E R	ggattttcatttagtttaaacaatctccctgcacatttcatttttttgc	
YO-2148	E225A/D227A F	gcacgtgaagatttctgcacctaattgcattgtcatgtttaaactggaaagtccccag	
YO-2149	E225A/D227A R	ctggggactccaggtaaaatgcacagcaatgcattgtgcagaaatctcacgtgc	
YO-2269	cGAS gRNA F	caccggaatgccaggggcgccccga	
YO-2270	cGAS gRNA R	aaactcgccggcccccgttgcattcc	
YO-2660	hCXCL10 F	gaaagcagttagcaaggaaaggt	34
YO-2661	hCXCL10 R	gacatatactccatgttagggaaagtga	34
YO-2772	Bsd F	taagcaccatggcaagcctttgtctca	
YO-2773	Bsd R	tgcattgcgttgcacttagccctccacacataac	
YO-2830	hMx1 F	accacagaggctctcagcat	10
YO-2831	hMx1 R	cagatcaggctcgtcaaga	10
YO-2834	hIL-1 β F	tacctgtccctgcgtgtgaa	78
YO-2835	hIL-1 β R	tcttggtaattttggatct	78
mHprtF	mHprt F	gttggatacaggccagacttttgt	

mHprtR	mHprt R	gagggtaggctggcctattggct
mUsp18F	mUsp18 F	cgtcggatgggtcatttg
mUsp18R	mUsp18 R	ggtcggatccacaacttc
mMx1F	mMx1 F	ccaactggaaatcctcctggaa
mMx1R	mMx1 R	gccgaccccttcctcatag
mRsad2F	mRsad2 F	aacaggctggttggagaag
mRsad2R	mRsad2 R	tgcattgctactatgctc

1010 *h, *Homo sapiens*; m, *Mus musculus*; F, forward; R, reverse

1011 ^U, Universal probe library

1012

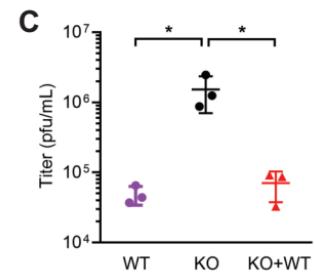
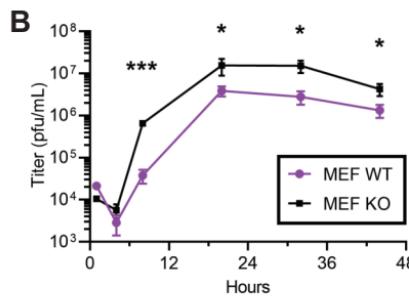
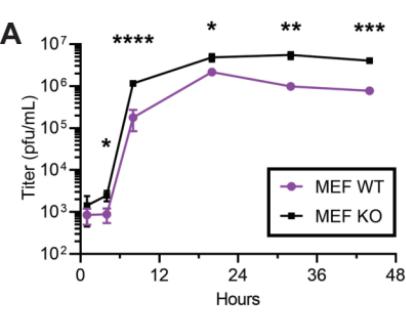
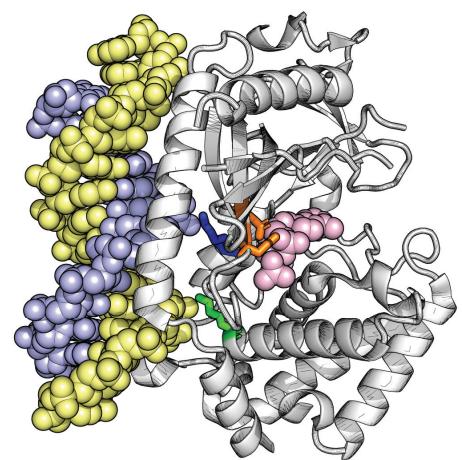
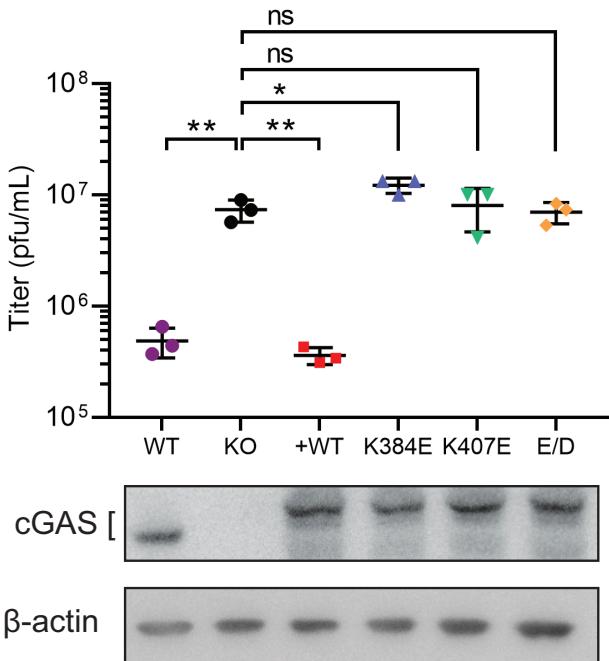
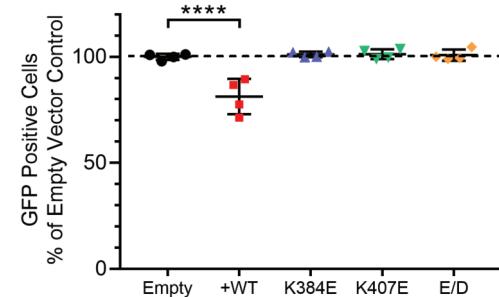
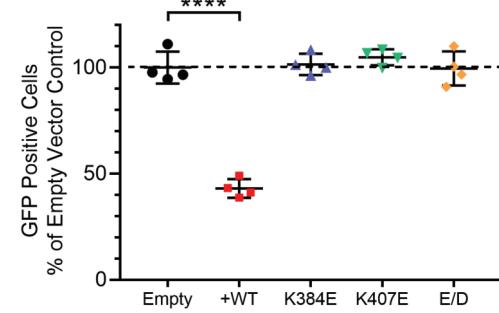
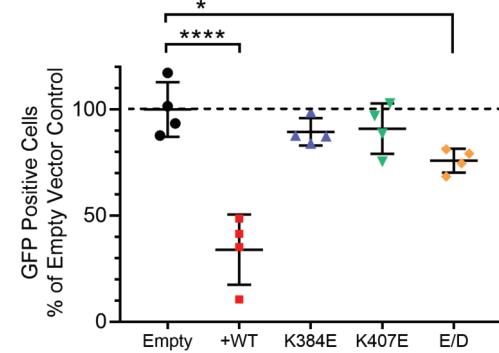
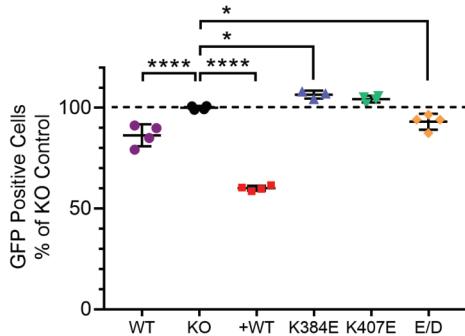
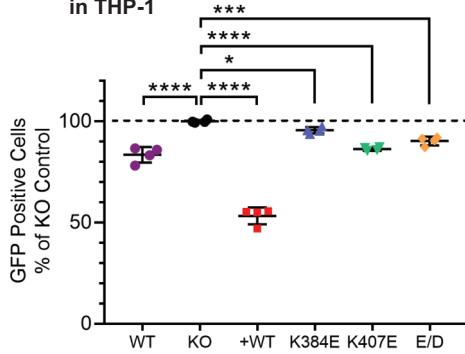
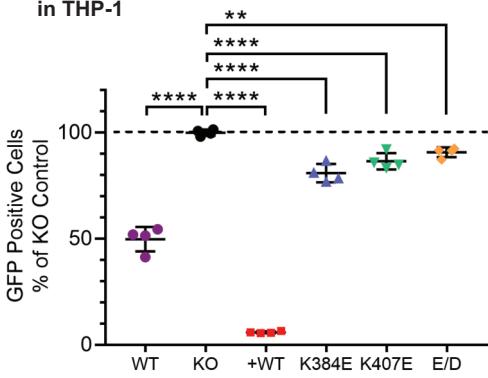


Figure 1

A**B****Figure 2**

A VSV-GFP in primary MEFs**B VSV Δ M51A-GFP in primary MEFs****C SINV-GFP in primary MEFs****D VSV-GFP in THP-1****E VSV Δ M51A-GFP in THP-1****F SINV-GFP in THP-1****Figure 3**

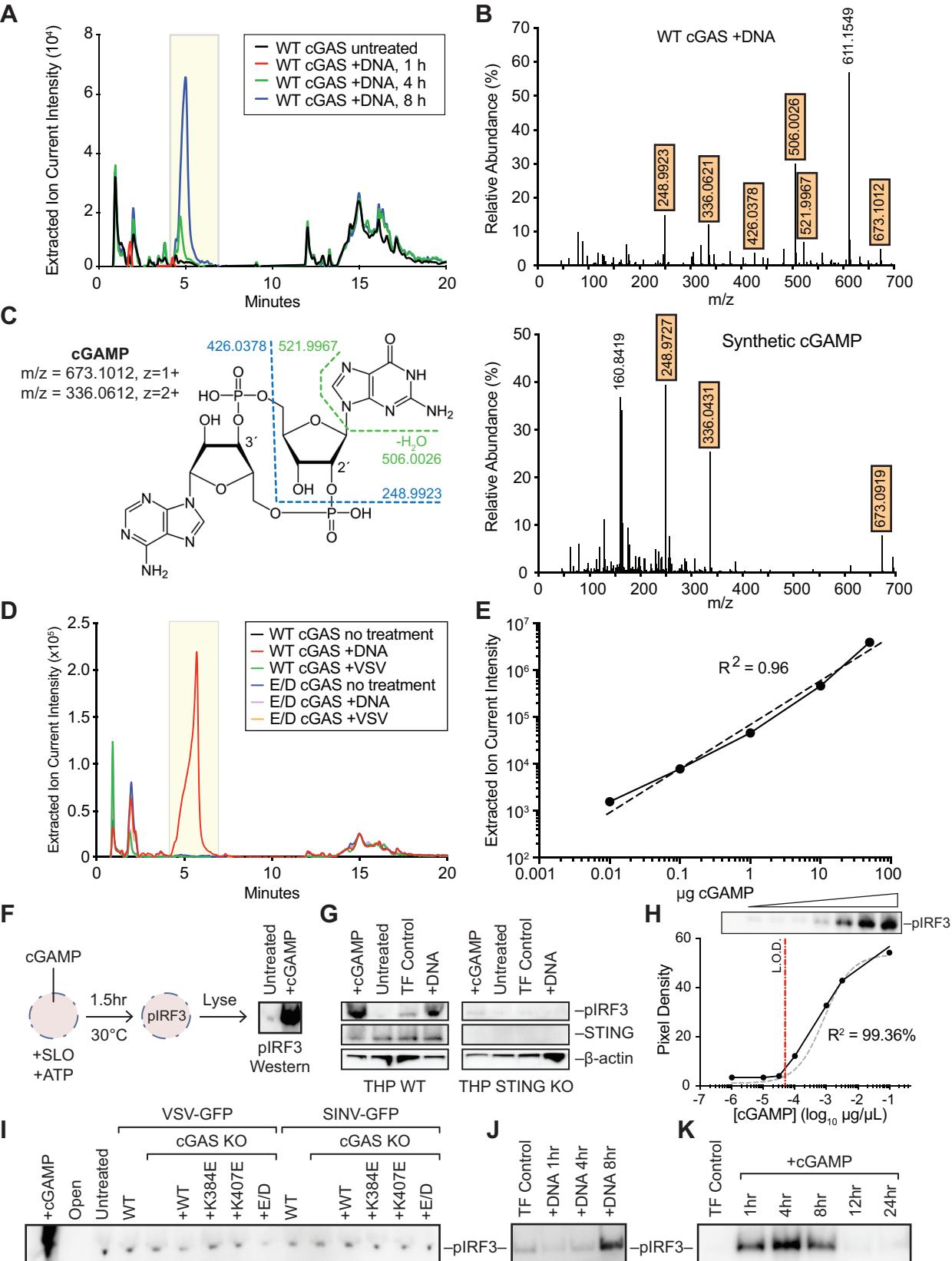


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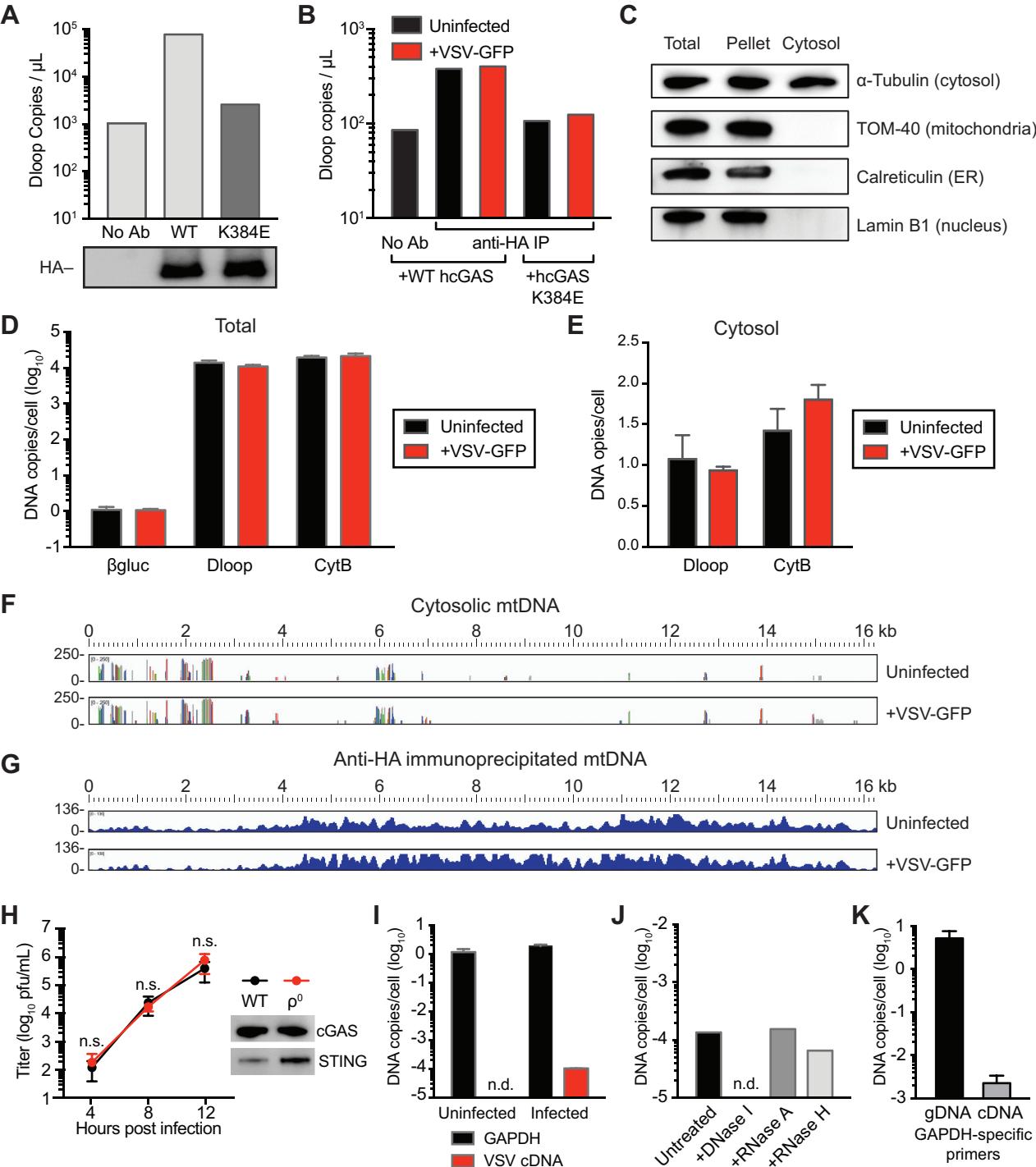


Figure 5

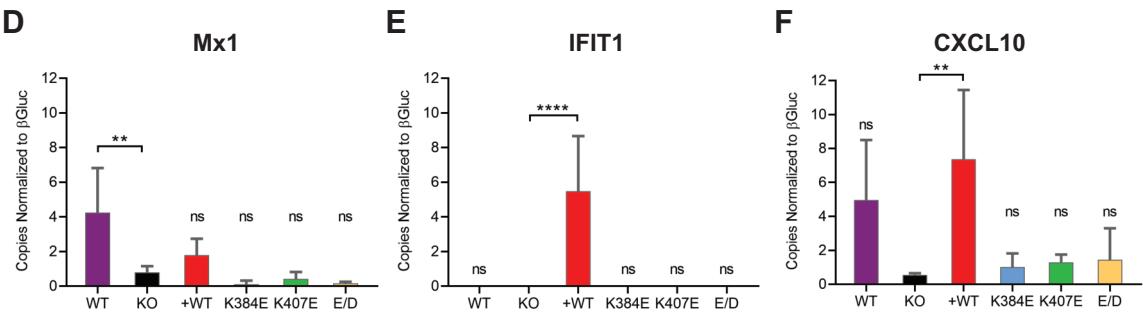
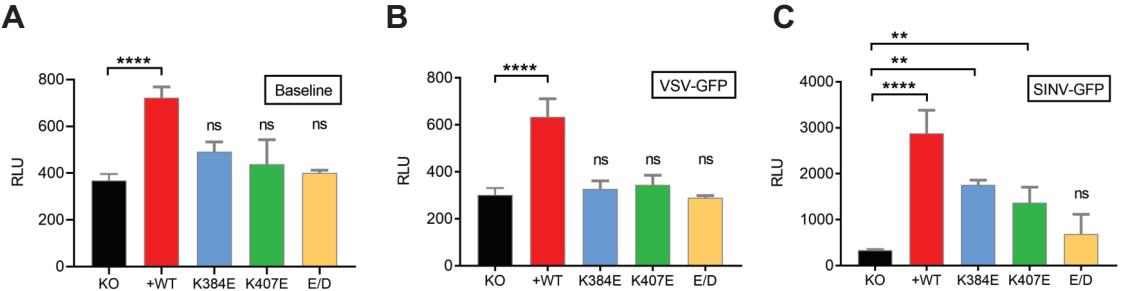


Figure 6

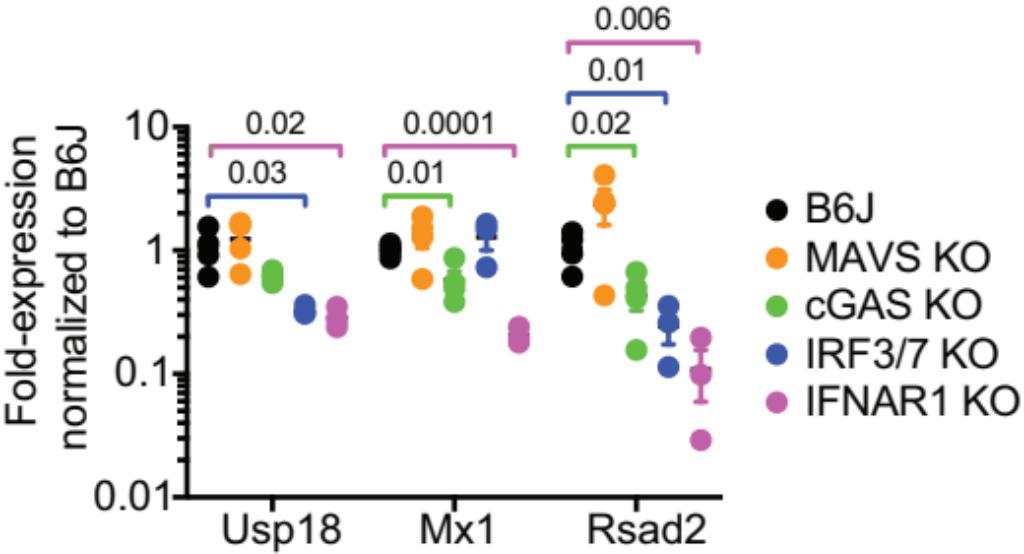


Figure 7

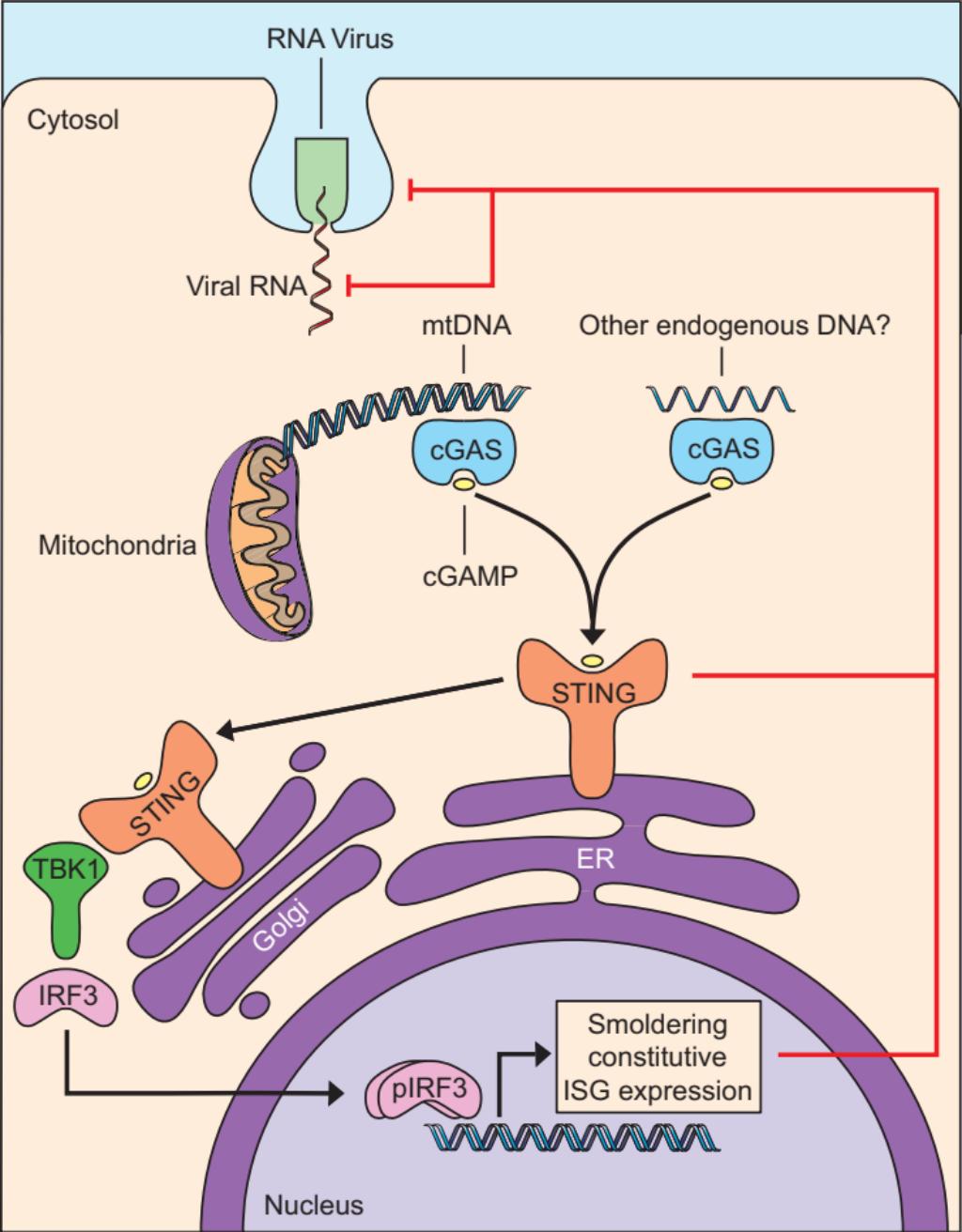


Figure 8