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deposited into the National Center for Biotechnology Information, NIH, Sequence Read Archive (accession number: SRP061783). E.S.S., W.L., and Stanford University have filed a provisional patent application. Supplementary materials contain additional data.

## SUPPLEMENTARY MATERIALS

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Materials and Methods  
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## ANTIVIRAL IMMUNITY

# Viruses transfer the antiviral second messenger cGAMP between cells

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Cyclic GMP–AMP synthase (cGAS) detects cytosolic DNA during virus infection and induces an antiviral state. cGAS signals by synthesis of a second messenger, cyclic GMP–AMP (cGAMP), which activates stimulator of interferon genes (STING). We show that cGAMP is incorporated into viral particles, including lentivirus and herpesvirus virions, when these are produced in cGAS-expressing cells. Virions transferred cGAMP to newly infected cells and triggered a STING-dependent antiviral program. These effects were independent of exosomes and viral nucleic acids. Our results reveal a way by which a signal for innate immunity is transferred between cells, potentially accelerating and broadening antiviral responses. Moreover, infection of dendritic cells with cGAMP-loaded lentiviruses enhanced their activation. Loading viral vectors with cGAMP therefore holds promise for vaccine development.

Type I interferons (IFNs) play pivotal roles in the immune response to virus infection (1). IFN expression is induced by signaling pathways activated by sensors of virus presence, including cytosolic DNA sensors (2, 3). Cyclic GMP–AMP synthase (cGAS) is a cytosolic DNA sensor that signals by catalyzing the synthesis of a second messenger, cyclic GMP–AMP (cGAMP) (4, 5). cGAMP binds to and activates stimulator of interferon genes (STING) (5, 6), which plays a central role in cytosolic DNA sensing by relaying signals from DNA sensors to transcription factors driving *IFN* gene transcription (3, 7).

DNA viruses and retroviruses trigger cGAS-dependent IFN responses in infected cells (8–15). This is thought to involve sensing by cGAS of viral DNA, leading to *IFN* gene transcription in the same cell where DNA detection occurred or in neighboring cells connected by gap junctions

(16). However, it is conceivable that IFN induction upon virus infection could also occur independently of cGAS if the infecting virus were to incorporate and transfer the cGAMP second messenger. For example, human immunodeficiency virus 1 (HIV-1) particles incorporate host molecules such as APOBEC3G (17). Given this precedent, we hypothesized that cGAMP can be packaged into virions and elicits an IFN response in newly infected cells independently of cGAS expression by the latter, allowing for potentiation of innate antiviral immunity.

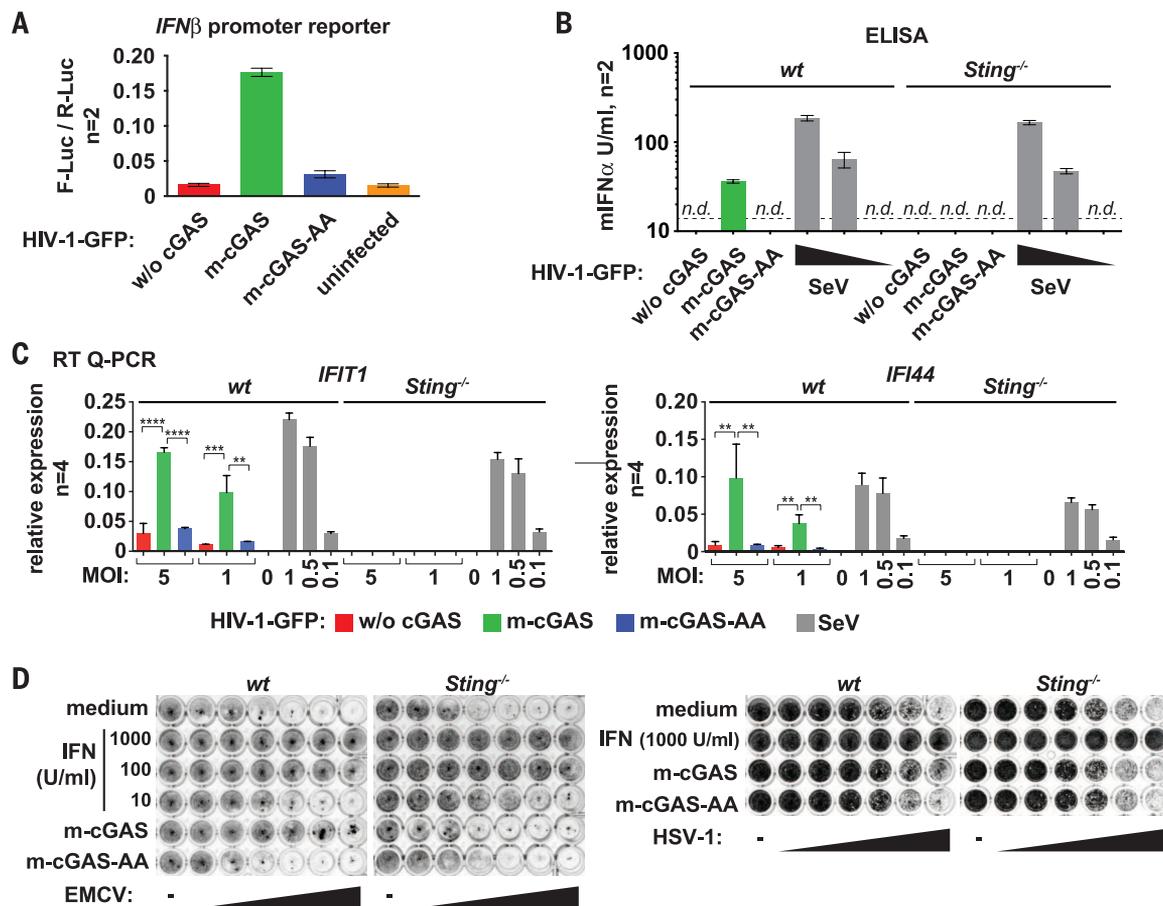
To test this idea, we produced HIV-1–based lentiviral vectors by plasmid transfection in 293T cells, a human cell line that does not express cGAS (4). Virus particles were pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), and the viral genome contained enhanced green fluorescent protein (EGFP) in the *Env* open reading frame. These viruses, henceforth referred to as HIV-1-GFP, are replication incompetent due to the lack of functional *Env*. Some 293T cells were cotransfected with expression constructs for either wild-type mouse cGAS (m-cGAS) or catalytically inactive m-cGAS-G198A/S199A (m-cGAS-AA) (4). Titrated virus stocks were then used to infect fresh human embryonic kidney 293 (HEK293) cells, which express endogenous

STING (7, 18) and induce IFN in response to cGAMP (fig. S1, A to D). HIV-1-GFP collected from cGAS-expressing cells triggered induction of an *IFN* $\beta$  promoter reporter, whereas viruses produced in the absence of exogenous cGAS or in the presence of mutant cGAS did not (Fig. 1A). Next, we analyzed IFN secretion by transferring supernatants from infected cells to a reporter cell line (HEK293-ISRE-luc), in which firefly luciferase expression is driven by interferon-stimulated response elements (ISREs) (fig. S1E). Only virus stocks produced in wild-type cGAS-expressing cells triggered IFN secretion (fig. S1F). Moreover, infected cells induced *IFI44* and *IFIT1* mRNAs specifically when cGAS was present in virus producer cells, further demonstrating induction of interferon-stimulated genes (ISGs) (fig. S1G). We made similar observations when infecting the myeloid cell line THP1 (fig. S2). Next, we infected primary mouse bone marrow–derived macrophages (BMDMs). IFN and ISGs were induced in BMDMs that had been infected with HIV-1-GFP produced in cGAS-reconstituted 293T cells (Fig. 1, B and C). STING-deficient BMDMs did not induce IFN and ISGs in response to the same virus preparations, although retinoic acid–inducible gene I (RIG-I)–dependent IFN production triggered by Sendai virus (SeV) was normal (Fig. 1, B and C). The increased IFN production triggered by HIV-1-GFP was functionally relevant, because infection with HIV-1-GFP produced in the presence of cGAS conferred a STING-dependent antiviral state against subsequent challenge with encephalomyocarditis virus (EMCV) or herpes simplex virus 1 (HSV-1) (Fig. 1D).

To exclude the possibility that transfer of plasmid DNA or of a soluble factor accounts for IFN production by freshly infected cells, we treated virus preparations with deoxyribonuclease or pelleted virions by centrifugation. Neither treatment affected the ability of HIV-1-GFP produced in cGAS-expressing cells to induce IFN (fig. S3, A and B). The IFN response in target cells was independent of reverse transcription and integration, as shown by pharmacological inhibition with nevirapine and raltegravir, respectively (fig. S3C). Virus-like particles lacking the viral RNA genome induced IFN in target cells when collected from cGAS-expressing producer cells (fig. S3D). These observations demonstrate that neither the viral genome nor its reverse transcription products account for IFN

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**Fig. 1. HIV-1-GFP produced in cGAS-reconstituted 293T cells induces IFN.** (A) HEK293 cells were transfected with p125-F-Luc (*IFN* $\beta$  promoter reporter) and pRL-TK. After 6 to 8 hours, cells were infected [multiplicity of infection (MOI) = 1] with HIV-1-GFP from producer cells expressing cGAS as indicated. F-Luc activity was analyzed after 24 hours and normalized to R-Luc. m-cGAS-AA is a catalytically inactive mutant ( $N = 2$  biological replicates, average and individual values are shown). (B) BMDMs of the indicated genotypes were infected with HIV-1-GFP (MOI = 5) or SeV (wedges: MOI=1, 0.5, 0.1). Supernatant was tested after 24 hours for mIFN $\alpha$  by enzyme-linked immunosorbent assay (ELISA) (n.d.: not detectable; dashed line: lower limit of de-

tection;  $N = 2$  biological replicates, average and individual values are shown). (C) BMDMs were infected as in (B) and the indicated mRNAs were quantified relative to *GAPDH* mRNA by reverse transcription-quantitative polymerase chain reaction (RT-QPCR) ( $N = 4$  replicates; error bars indicate SD, and significance was determined by analysis of variance (ANOVA); \*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ). (D) Lung fibroblasts were treated with IFN-A/D or infected with HIV-1-GFP (MOI = 2). Cells were then infected with EMCV or HSV-1 (wedges: fourfold dilutions starting at MOI = 4 or MOI = 64, respectively). After 24 hours, cells were stained with crystal violet. Data are representative of three or more independent experiments.

duction in this setting. Substitution of VSV-G with thogotovirus glycoprotein did not diminish the IFN-inducing property of virus stocks from cGAS-expressing cells, showing that these effects are not related to VSV-G pseudotyping (fig. S3E) (19).

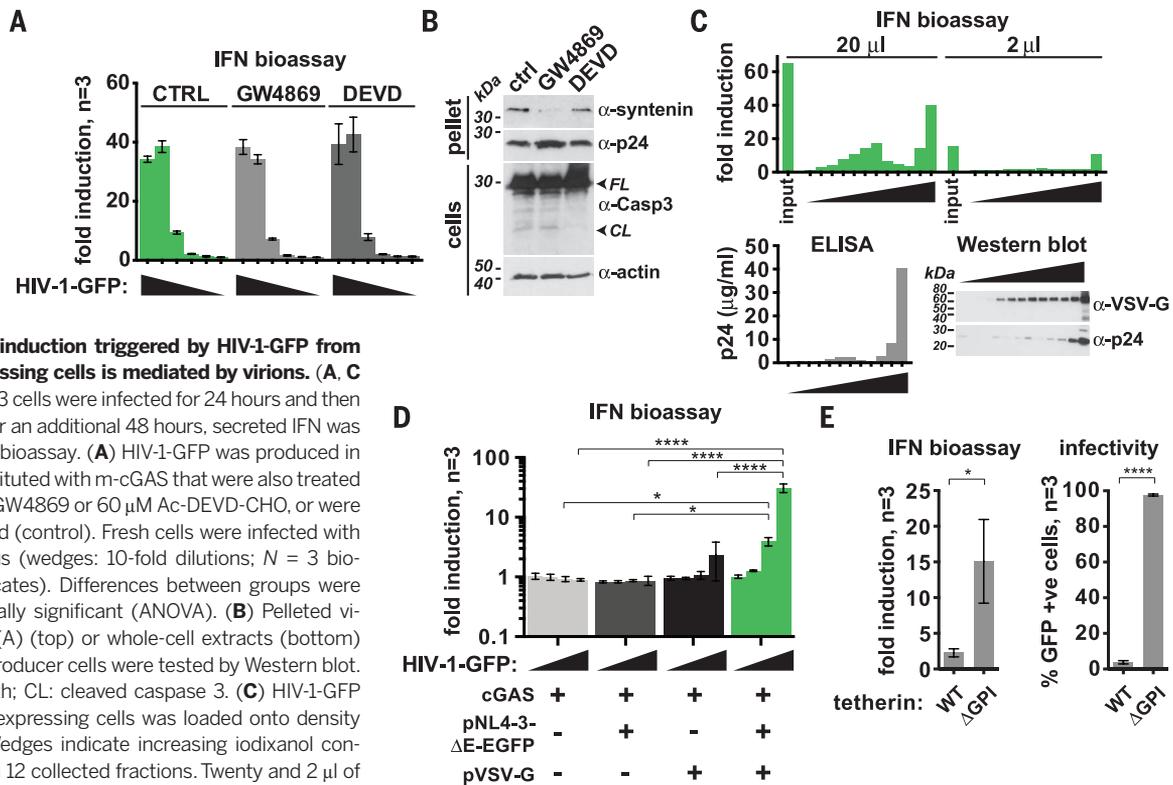
HIV-1-GFP stocks are likely to contain exosomes and other enveloped vesicles such as apoptotic bodies. Treatment of cGAS-reconstituted producer cells with the exosome inhibitor GW4869 (20) or the caspase inhibitor Ac-DEVD-CHO during virus preparation did not affect IFN induction by HIV-1-GFP (Fig. 2A). Reduced amounts of the exosome marker syntenin (21) in virus preparations and diminished cleavage of caspase 3 in virus producer cells confirmed the efficacy of these compounds (Fig. 2B). We then separated virions from exosomes and other extracellular vesicles by density gradient centrifugation (22) and found that the fraction containing most of the viral p24 protein and of VSV-G was also the most potent fraction

inducing IFN (Fig. 2C). Next, we transfected 293T cells with cGAS plasmid alone and prepared mock “virus” stocks. These preparations did not induce detectable amounts of IFN (Fig. 2D), suggesting that vesicles constitutively shed by cells do not account for IFN induction. Moreover, overexpression of tetherin, which inhibits virion release (23), diminished IFN induction (Fig. 2E). Together, these observations show that most of the IFN-inducing activity is associated with virions.

Next, we prepared small-molecule extracts from HIV-1-GFP, which we then added to PMA (phorbol 12-myristate 13-acetate)-differentiated THP1 cells that were mildly permeabilized with digitonin (fig. S4). HIV-1-GFP extracts collected from wild-type cGAS-reconstituted producer cells induced IFN secretion, whereas HIV-1-GFP extracts collected from catalytically inactive m-cGAS-AA-reconstituted producer cells failed to induce IFN secretion (Fig. 3A). Preincubation of extracts with snake venom phosphodiesterase I

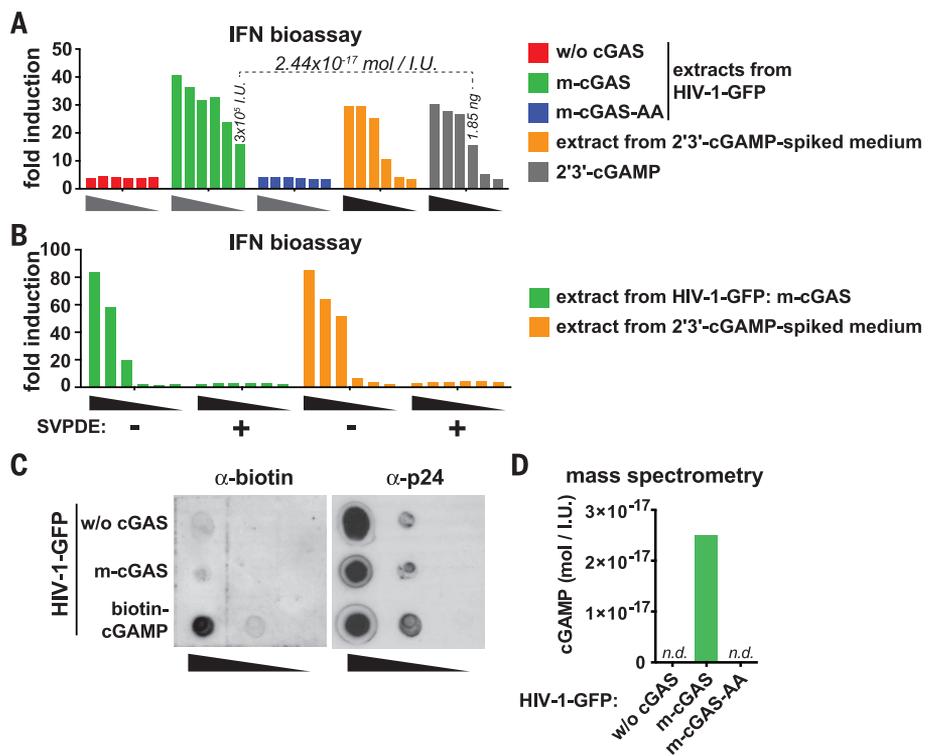
(SVPDE), which cleaves cGAMP (24), abrogated this effect (Fig. 3B). To further test whether cGAMP is present in lentiviral particles, we transfected virus producer cells with biotin-labeled cGAMP. Biotin-cGAMP was detectable in virus preparations (Fig. 3C). Next, we quantified the amount of cGAMP present in HIV-1-GFP preparations using mass spectrometry. cGAMP was detectable only in extracts from virus produced in cGAS-expressing cells (Fig. 3D). Based on a calibration curve and the efficiency of extraction (fig. S5), we found that  $2.50 \times 10^{-17}$  mol of cGAMP were present in virus stocks per infectious unit (Fig. 3D). A similar estimate was obtained from the data in Fig. 3A.

These results show that cGAMP can be packaged into HIV-1-GFP and induces IFN via STING in newly infected cells. Similarly, we found that an HIV-2-based, replication-deficient lentivirus produced in cGAS-expressing cells induced IFN secretion in HEK293 cells (fig. S6). To further



**Fig. 2. IFN induction triggered by HIV-1-GFP from cGAS-expressing cells is mediated by virions.** (A, C to E) HEK293 cells were infected for 24 hours and then washed; after an additional 48 hours, secreted IFN was analyzed by bioassay. (A) HIV-1-GFP was produced in cells reconstituted with m-cGAS that were also treated with 20 μM GW4869 or 60 μM Ac-DEVD-CHO, or were left untreated (control). Fresh cells were infected with pelleted virus (wedges: 10-fold dilutions; N = 3 biological replicates). Differences between groups were not statistically significant (ANOVA). (B) Pelleted viruses from (A) (top) or whole-cell extracts (bottom) from virus producer cells were tested by Western blot. FL: full length; CL: cleaved caspase 3. (C) HIV-1-GFP from cGAS-expressing cells was loaded onto density gradients. Wedges indicate increasing iodixanol concentration in 12 collected fractions. Twenty and 2 μl of each fraction and of the input were tested. p24 concentrations were determined by ELISA (bottom left) and p24 and VSV-G were analyzed by Western blot (bottom right). (D) 293T cells were transfected as indicated to produce mock “virus” stocks (wedges: 10-fold dilutions; N = 3 biological replicates). (E) Vpu-deficient lentivectors produced in cells expressing cGAS and either wild-type or mutant tetherin were tested. The fraction of infected cells was determined by flow cytometry (right). N = 3 biological replicates. Error bars are ±SD, and significance was determined by ANOVA (A and D) or unpaired t test (E) (\*\*\*\*P < 0.0001; \*P < 0.05). Data are representative of two (C) or three (A, B, D, and E) independent experiments.

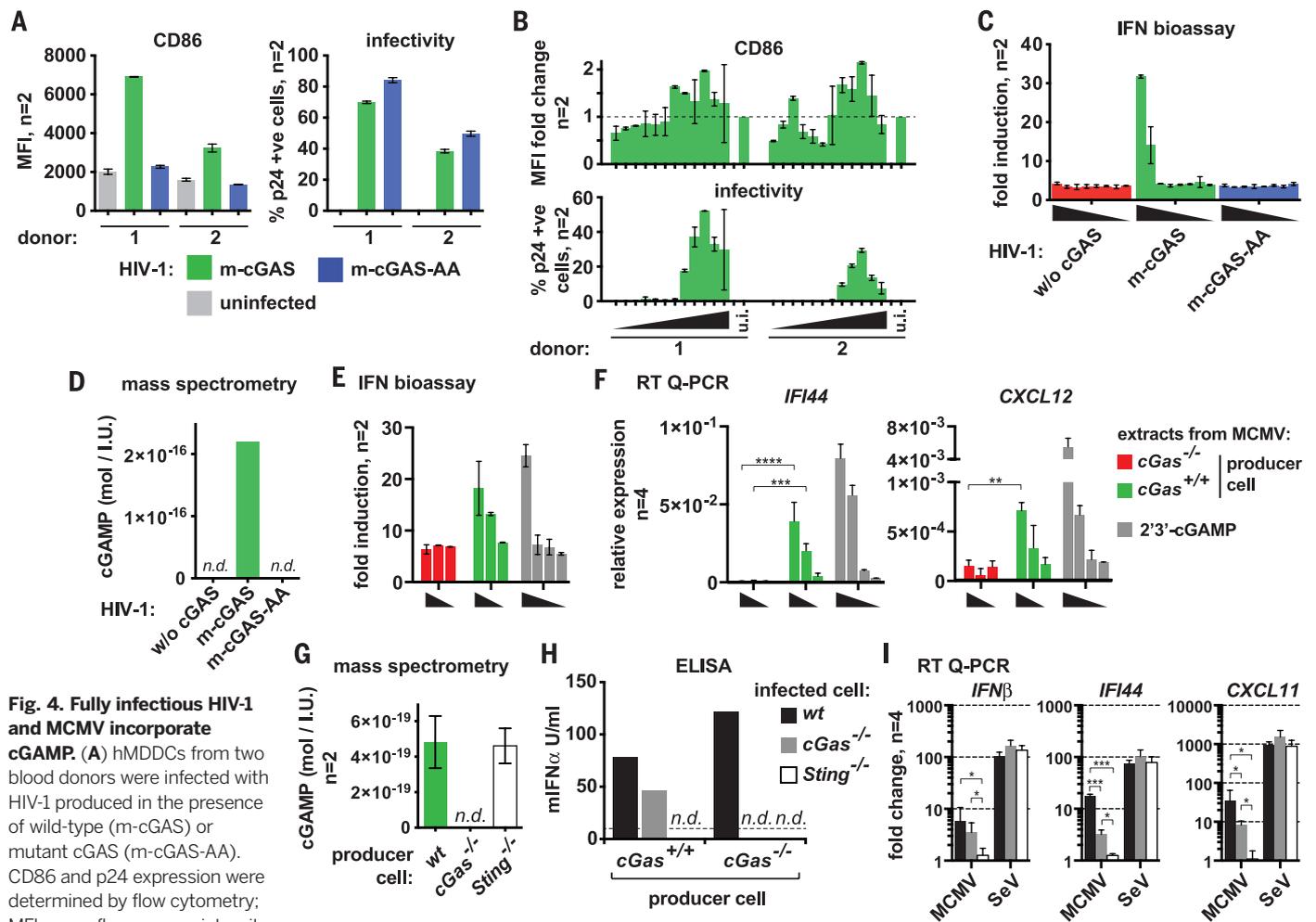
**Fig. 3. Small-molecule extracts from HIV-1-GFP generated in cGAS-reconstituted producer cells induce IFN and contain cGAMP.** (A) Extracts from viruses produced in the absence of cGAS or in the presence of wild-type (m-cGAS) or mutant cGAS (m-cGAS-AA) were added to digitonin permeabilized THP1 cells. IFN in THP1 supernatants was assessed by bioassay. Gray wedges represent a 1:2 dilution series starting with extract from 10<sup>7</sup> infectious units. As controls, synthetic 2'3'-cGAMP was either directly added to THP1 cells (gray bars) or was spiked into medium and then included in the extraction procedure (orange bars). Black wedges represent a 1:3 dilution series starting with 50 ng of 2'3'-cGAMP. Extraction efficiency (fig. S5B) was taken into account to estimate the amount of cGAMP per infectious unit (dotted line). (B) Extract from 10<sup>7</sup> infectious units of HIV-1-GFP produced in the presence of cGAS was incubated with or without SVPDE for 1 hour and then added to digitonin-permeabilized THP1 cells. IFN in THP1 supernatants was assessed by bioassay. Wedges represent a 1:3 dilution series. (C) HIV-1-GFP produced in the absence or presence of cGAS or in biotin-cGAMP-transfected cells was probed by dot blot for biotin (left). The stripped membrane was reprobed for p24 (right). Wedges represent a 1:10 dilution series starting with 2 × 10<sup>6</sup> infectious units. (D) cGAMP concentration in samples from (A) was analyzed by mass spectrometry. n.d., not detectable. Data are representative of two (B) or three (A, C, D) independent experiments.



explore the relevance of our findings, we extended our study from replication-deficient, VSV-G-pseudotyped lentiviruses to fully infectious HIV-1 bearing a native CXCR4-tropic envelope glycoprotein. Human monocyte-derived dendritic cells (hMDDCs) infected with HIV-1 that had been produced in 293T cells reconstituted with wild-type cGAS induced the expression of IFN and of CD86, encoded by an ISG (Fig. 4A and figs. S7 to S9). Density gradient centrifugation demonstrated that most of the CD86-inducing activity fractionated together with infectious virus particles (Fig. 4B). Next, we prepared extracts and found that these induced IFN secretion by THP1 cells (Fig. 4C). Mass spec-

trometry confirmed that cGAMP was specifically present in extracts from HIV-1 stocks from cGAS-expressing cells (Fig. 4D). These results show that cGAMP is not only incorporated into VSV-G-pseudotyped lentivectors but can also be incorporated into fully infectious HIV-1. However, these experiments relied on overexpression of cGAS in virus producer cells. We therefore asked whether triggering of endogenous cGAS during virus infection results in the packaging of cGAMP into progeny virus particles. We used mouse cytomegalovirus (MCMV), an enveloped DNA virus, for these experiments (fig. S10) because this virus induces STING-dependent IFN responses (25). We propagated MCMV in primary

mouse embryonic fibroblasts (MEFs). Extracts from MCMV stocks collected from wild-type cells contained an activity that induced IFN secretion and ISG expression in THP1 cells, whereas extracts from virus from cGAS-deficient cells did not (Fig. 4, E and F). Mass spectrometry confirmed the presence of cGAMP in MCMV stocks from wild-type cells (Fig. 4G). Similarly, virus produced in *Sting*<sup>-/-</sup> cells contained cGAMP, whereas no cGAMP was detectable in virus preparations from *cGas*<sup>-/-</sup> cells (Fig. 4G). Consistent with this observation, MCMV produced in cGAS-sufficient MEFs induced IFN $\alpha$  secretion by *cGas*<sup>-/-</sup> BMDMs upon infection, but failed to do so in *Sting*<sup>-/-</sup> cells (Fig. 4H). In contrast, the response to MCMV



fectious units or 0.0686 ng of cGAMP. (G) cGAMP concentrations in MCMV extracts were analyzed by mass spectrometry. n.d., not detectable. Data were pooled from two independent experiments. Average and individual values are shown. (H) BMDMs of the indicated genotypes were infected with MCMV (MOI = 7) from *cGas*<sup>+/+</sup> or *cGas*<sup>-/-</sup> MEFs, and supernatant mIFN $\alpha$  was analyzed by ELISA after 20 hours (n.d., not detectable; dashed line: lower limit of detection). (I) BMDMs as in (H) were infected with MCMV (MOI = 1) from *cGas*<sup>+/+</sup> MEFs for 20 hours. The indicated mRNAs were quantified by RT-QPCR relative to *GAPDH* mRNA, and fold changes compared to mock infected cells were calculated ( $N = 4$  replicates). SeV was used as a control (MOI = 0.5). In (F) and (I), error bars indicate SD, and significance was determined by ANOVA (\*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ). Data are representative of three (A) or two (B to F, I) independent experiments.

mouse embryonic fibroblasts (MEFs). Extracts from MCMV stocks collected from wild-type cells contained an activity that induced IFN secretion and ISG expression in THP1 cells, whereas extracts from virus from cGAS-deficient cells did not (Fig. 4, E and F). Mass spectrometry confirmed the presence of cGAMP in MCMV stocks from wild-type cells (Fig. 4G). Similarly, virus produced in *Sting*<sup>-/-</sup> cells contained cGAMP, whereas no cGAMP was detectable in virus preparations from *cGas*<sup>-/-</sup> cells (Fig. 4G). Consistent with this observation, MCMV produced in cGAS-sufficient MEFs induced IFN $\alpha$  secretion by *cGas*<sup>-/-</sup> BMDMs upon infection, but failed to do so in *Sting*<sup>-/-</sup> cells (Fig. 4H). In contrast, the response to MCMV

from cGAS-deficient producer cells required both cGAS and STING. Furthermore, *IFN $\beta$* , ISG, and chemokine mRNA up-regulation after infection with MCMV from wild-type producer cells was partially cGAS-independent but fully STING-dependent (Fig. 4I). Taken together, these results demonstrate that cGAMP produced by endogenous cGAS is incorporated into MCMV particles, which contributes to IFN induction in newly infected cells in a cGAS-independent but STING-dependent manner.

Incorporation of cGAMP into virions may broaden the spectrum of cells that initiate an IFN response. For example, HIV-1 reverse transcription, which is inhibited in some cells by SAMHD1 (26), is not required for virus sensing via cGAMP transfer. This Trojan horse mechanism may also accelerate the IFN response. We speculate that cGAMP packaging had been overlooked in previous studies owing to virus production in cGAS-negative cell lines such as 293T. Whether the incorporation of cGAMP into virus particles is a selective process or is based on diffusion remains to be determined. It is likely that a lipid envelope is required to encompass cGAMP in virus particles. Indeed, nonenveloped adenovirus produced in cGAS-reconstituted cells did not induce IFN in newly infected cells (fig. S11). In our experimental settings, cGAMP was primarily transferred by virions; however, these data do not exclude a role of extracellular vesicles such as exosomes in shuttling cGAMP between cells in other models (for example, sterile inflammation). Although it remains unclear whether HIV virions produced during natural infection contain appreciable amounts of cGAMP, our observations have important translational implications. cGAMP delivery via lentiviral vectors results in heightened activation of DCs (figs. S8, S9, and S12). This could be harnessed in vaccination settings in which viral vectors codeliver to DCs cyclic dinucleotides as innate stimuli, as well as viral antigens. Moreover, IFN induction by oncolytic viruses has been reported to enhance tumor killing (27) and may be achieved by cGAMP loading. In sum, we have identified a mechanism by which a signal for innate immunity is transferred between cells.

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#### SUPPLEMENTARY MATERIALS

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#### ANTIVIRAL IMMUNITY

# Transmission of innate immune signaling by packaging of cGAMP in viral particles

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**Infected cells detect viruses through a variety of receptors that initiate cell-intrinsic innate defense responses. Cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS) is a cytosolic sensor for many DNA viruses and HIV-1. In response to cytosolic viral DNA, cGAS synthesizes the second messenger 2'3'-cyclic GMP-AMP (cGAMP), which activates antiviral signaling pathways. We show that in cells producing virus, cGAS-synthesized cGAMP can be packaged in viral particles and extracellular vesicles. Viral particles efficiently delivered cGAMP to target cells. cGAMP transfer by viral particles to dendritic cells activated innate immunity and antiviral defenses. Finally, we show that cell-free murine cytomegalovirus and Modified Vaccinia Ankara virus contained cGAMP. Thus, transfer of cGAMP by viruses may represent a defense mechanism to propagate immune responses to uninfected target cells.**

**T**o protect multicellular organisms against viruses, it is vital that infected cells trigger antiviral defense responses that can be rapidly transmitted to noninfected cells. Cells are equipped with cytosolic viral sensors that recognize viral infection and induce innate immune responses (1). The resulting innate immune responses can restrain the pathogen, repair the host, and shape an adaptive immune response (2). Cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS) is a cytosolic DNA sensor that synthesizes 2'3'-cyclic GMP-AMP (cGAMP), a second messenger that binds to the STING protein and activates a type I interferon (IFN) response (3–5). cGAS is essential to induce an antiviral response against several DNA viruses, as well as HIV (6–8).

Dendritic cells (DCs) link innate sensing of pathogens to induction of adaptive immune responses. In DCs, the cGAS-cGAMP pathway also activates the expression of cytokines and costimulatory molecules for T cell activation, similar to adjuvants (7, 9). In human DCs, HIV-1 normally evades sensing by cGAS, suggesting a crucial

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## Viruses transfer the antiviral second messenger cGAMP between cells

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### Viruses pack antiviral mediators

Viruses often hijack host proteins for their own use, turning host cells into virion-spewing machines. However, Bridgeman *et al.* and Gentili *et al.* now report a sneaky way that the host can fight back (see the Perspective by Schoggins). Host cells that expressed the enzyme cGAS, an innate immune receptor that senses cytoplasmic DNA, packaged the cGAS-generated second messenger cGAMP into virions. Virions could then transfer cGAMP to neighboring cells, triggering an antiviral gene program in these newly infected cells. Such transfer of an antiviral mediator may help to speed up the immune response to put the brakes on viral spread.

*Science*, this issue pp. 1228 and 1232; see also p. 1166

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