Lysyl-tRNA synthetase produces diadenosine tetraphosphate to curb STING-dependent inflammation

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Inflammation is an essential part of immunity against pathogens and tumors but can promote disease if not tightly regulated. Self and non-self-nucleic acids can trigger inflammation, through recognition by the cyclic GMP-AMP (cGAMP) synthetase (cGAS) and subsequent activation of the stimulator of interferon genes (STING) protein. Here, we show that RNA:DNA hybrids can be detected by cGAS and that the Lysyl-tRNA synthetase (LysRS) inhibits STING activation through two complementary mechanisms. First, LysRS interacts with RNA:DNA hybrids, delaying recognition by cGAS and impeding cGAMP production. Second, RNA:DNA hybrids stimulate LysRS-dependent production of diadenosine tetraphosphate (Ap4A) that in turn attenuates STING-dependent signaling. We propose a model whereby these mechanisms cooperate to buffer STING activation. Consequently, modulation of the LysRS-Ap4A axis in vitro or in vivo interferes with inflammatory responses. Thus, altogether, we establish LysRS and Ap4A as pharmacological targets to control STING signaling and treat inflammatory diseases.

INTRODUCTION

Dinucleotides are bioactive molecules for which a signaling role in mammalian cells has emerged in recent years. In particular, cyclic dinucleotides, such as cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), have been described as activators of the inflammatory response (1). In the presence of self- or non-self–nucleic acid species, the cyclic GMP-AMP synthase (cGAS) DNA sensor produces cGAMP (2), activating inflammatory responses (3). A pivotal step in the mounting of this response to immune-stimulatory nucleic acids is the interaction of cGAMP with the stimulator of interferon genes (STING) (4), which leads to the recruitment of the tank binding kinase 1 (TBK1) and transcription factors, including interferon response factor 3 (IRF3) (5). The assembly of this STING signalosome leads to the phosphorylation of IRF3 that translocates into the nucleus to orchestrate the transcription of a set of genes that includes proinflammatory cytokines and type I interferon (IFN) (6). Dysregulation of the cGAS-STING pathway fuels several inflammatory human pathologies, including autoimmune, autoinflammatory, and malignant disorders (7). The development of means to act on the cGAS-STING signaling pathway is therefore paramount to current therapies aiming to curb disease-associated inflammation (8). Small molecules that modulate cGAS-STING pathway activation have been identified (9), opening novel investigation avenues. Yet, in certain pathologies, what determines the outcome of STING activation or inhibition remains unknown (10).

This underscores the pressing need for a better understanding of the mechanisms regulating the cGAS-STING pathway in different cellular contexts.

Cytosolic double-stranded DNA (dsDNA) species are the main activators of the cGAS-STING pathway, but recently, RNA:DNA hybrids have also been described as possible ligands for cGAS. However, the molecular mechanisms involved in their recognition and in the regulation of ensuing inflammatory cytokines production are poorly described. RNA:DNA hybrids can arise as by-products of several physiological processes, including RNA replication, immunoglobulin class switching and hypermutation, or double-strand DNA break repair (11). Endogenous and exogenous reverse transcripts also contribute to generating RNA:DNA hybrids (12, 13). Regardless of their origin, these moieties can threaten cellular integrity, by promoting unwanted triggering of inflammatory responses (12–14). However, while RNA:DNA hybrids can stimulate cGAS-dependent cGAMP production in vitro (14), it is still unknown whether such interaction can occur in cells.

To identify proteins involved in the detection of RNA:DNA hybrids and in the regulation of associated inflammatory responses, we used a biochemistry approach. We thereby identified the Lysyl tRNA synthetase (LysRS), which is a component of the cytosolic multi-tRNA synthetase complex (MSC), as a direct binding partner of RNA:DNA hybrids. While LysRS plays major roles in translation through aminoacylation of the cognate tRNA, noncanonical functions have been described (15, 16), including regulation of immune response (17–19). This prompted us to investigate the role of LysRS in detection and regulation of RNA:DNA hybrid–induced inflammatory responses. We thus demonstrate that RNA:DNA hybrids can be detected by cGAS and that LysRS can regulate subsequent STING activation through two complementary mechanisms. On one hand, LysRS interacts with cytosolic RNA:DNA hybrids, delaying recognition by cGAS. On the other hand, stimulation of cells with RNA:DNA hybrids leads to LysRS-dependent diadenosine tetraphosphate (Ap4A) production. This second messenger can associate with STING, delaying its activation. We therefore show that the LysRS-Ap4A pathway
contributes to regulate nucleic acid-dependent cGAS-STING activation.

RESULTS

The MSC interacts with RNA:DNA hybrids

To identify RNA-DNA interacting proteins, we used a blunt synthetic 40-nt-long RNA:DNA hybrid bearing a 5′ biotin (B RNA:DNA; fig. S1A). We first verified the stability of synthetic RNA:DNA hybrids by immunofluorescence (fig. S1B) in mouse embryonic fibroblasts (MEFs), together with their ability to elicit a proinflammatory response in MEF, MEF knocked out for Sting (MEF<sup>Sting<sup>−/−</sup></sup>) or cGAS (MEF<sup>cGAS<sup>−/−</sup></sup>). The latter was assessed by measuring Ifnβ mRNA levels by real-time quantitative polymerase chain reaction (RT-qPCR) (Fig. 1A) and IRF3 mRNA levels by real-time quantitative RT-PCR (Fig. 1A). Ifnβ and IRF3 mRNA levels increased upon transfection of RNA:DNA hybrids in human (Fig. 1E) and murine cells (Fig. 1D). We recapitulated the specific interaction of the MSC with RNA:DNA hybrids in human (Fig. 1E) and murine cells (Fig. 1D) upon transfection of RNA:DNA hybrids and streptavidin pull-down. We therefore show that the MSC is recruited to RNA:DNA hybrids both in vitro and in cells.

The Lysyl tRNA synthetase interacts directly with RNA:DNA hybrids

We next investigated which member of the MSC is responsible for its interaction with RNA:DNA hybrids. Three members of the MSC complex, namely, LysRS, AspRS, and p43, comprise oligonucleotide/oligosaccharide binding (OB)–fold domains that are predicted to bind nucleic acids (21). Thus, we tested whether these proteins can directly interact with RNA:DNA hybrids using recombinant glutathione S-transferase (GST)–tagged proteins (fig. S2A). GST was used as a control. We incubated purified proteins with RNA:DNA hybrids before pull-down using streptavidin affinity beads and analysis by WB using an anti-GST antibody. We thereby show that LysRS displays strong binding to RNA:DNA hybrids (Fig. 2A) and that this interaction is Ribonuclease H (RNaseH)–sensitive (Fig. 2B). We further characterized the binding of LysRS to RNA:DNA hybrids, using full-length LysRS or LysRS constructs harboring either a N-terminal deletion of the putative OB-fold nucleic acid binding domain (LysRS<sup>N</sup>) or a C-terminal deletion of the catalytic aminoacyl transferase domain (LysRS<sup>C</sup>) (22). Recombinant GST-LysRS, GST-LysRS<sup>N</sup>, GST-LysRS<sup>C</sup>, and GST (fig. S2B) were used in in vitro pull-down experiments using RNA:DNA hybrids immobilized on streptavidin affinity beads. This showed that LysRS interacts with RNA:DNA hybrids through its N-terminal domain (Fig. 2C). This binding is sensitive to RNaseH digestion, indicating that the binding of LysRS to RNA:DNA hybrids is Ribonuclease H (RNaseH)–sensitive (Fig. 2B).

Fig. 1. MSC is recruited to RNA:DNA hybrids. (A) Mean (±SEM), Ifnβ mRNA levels in WT-MEF, MEF<sup>Sting<sup>−/−</sup></sup>, and MEF<sup>cGAS<sup>−/−</sup></sup> transfected or not with RNA:DNA hybrids (R:D); n = 8. t test, ****P < 0.0001. (B) WB analysis of whole-cell extracts of cells treated as in (A). Membranes were probed with the indicated antibodies. (C) Left: Experimental scheme. M<sub>5</sub>, mass spectrometry; ss<sup>RNA</sup>, biotinylated ss<sup>RNA</sup>; ss<sup>R:D</sup>, biotinylated RNA:DNA hybrids. Right: Silver staining of samples obtained following the experimental scheme. Numbers indicate molecular weight (MW) in kDa. (D) WB analysis of pull-down performed as in (C), except that biotinylated ss<sup>RNA</sup> (ss<sup>RNA</sup>) was included as a control. (E) HeLa cells were transfected or not with biotinylated RNA:DNA hybrids before pull-down assay by incubating cytosolic extracts (S100) from HeLa-S3 cells (fig. S1C) with B RNA:DNA hybrids before pull-down using streptavidin affinity beads. Input and eluates were analyzed by WB using the indicated antibodies. All immunoblots show representative experiments.

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to RNaseH treatment (Fig. 2D). We additionally confirmed that murine LysRS (fig. S2B) binds RNA:DNA hybrids (fig. S2C) and that this binding is RNaseH sensitive (fig. S2D) as observed for human LysRS (Fig. 2, A to C). Thus, our in vitro experiments shown that LysRS can directly bind RNA:DNA hybrids.

Our in vitro binding data further suggest that LysRS may be the key to the recruitment of MSC proteins to RNA:DNA hybrids. To test this, we first FLAG-purified overexpressed FLAG-hemagglutinin (HA)–tagged LysRS (F/HA-LysRS) from the cytosolic fraction of HeLa cells. Eluted material was used as input for pull-down using streptavidin-immobilized RNA:DNA hybrids (Fig. 2E, left). WB analysis using antibodies targeting proteins of the MSC complex revealed that all tested members coprecipitated with RNA:DNA hybrids (Fig. 2E, right). We confirmed the specificity of this interaction by RNaseH treatment of the RNA:DNA hybrids before binding to the streptavidin affinity beads (Fig. 2E, right). We next knocked down LysRS in cells before pull-down assays using RNA:DNA hybrids. This showed that decreased levels of LysRS (Fig. 2F, compare lanes 1 and 2) are accompanied by decreased association of other tested components of the MSC (Fig. 2F, compare lanes 4 and 6). This indicates that the presence of LysRS is the key to the recruitment of the MSC to RNA:DNA hybrids in cells. Last, we questioned whether LysRS interacts with endogenous RNA:DNA hybrids. To this aim, we adapted the S9.6 antibody–based DNA:RNA hybrid immunoprecipitation protocol [adapted from (13)]. Using the HeLa cell line that presents endogenous RNA:DNA hybrids (fig. S2E), we confirmed that endogenous RNA:DNA hybrids immunoprecipitated LysRS (Fig. 2G). This was further confirmed by experiments where endogenous GlnRS was immunopurified, before assessment of the presence of other components of the MSC and extraction of GlnRS–associated DNA (fig. S2F). WB analysis of the eluates confirmed the presence of LysRS (fig. S2F, middle), and quantitative PCR analysis showed an enrichment of sequences belonging to the high-copy satellite II region of the human genome (fig. S2F, right) that is reported to produce RNA:DNA hybrids (23). Thus, together, our data show that within the MSC complex LysRS is the main mediator of the interaction with RNA:DNA hybrids.

**The Lysyl tRNA synthetase inhibits RNA:DNA hybrid-induced IFN production**

While the main reported function of LysRS within the MSC complex is to aminocylate its cognate tRNA, there are reports of additional noncanonical functions, including negative regulation of immune responses (15, 16). Thus, we interrogated whether LysRS can modulate RNA:DNA hybrid–associated inflammation. To this aim, we transduced WT-MEF with a LysRS targeting short hairpin RNA (shRNA) (shLysRS) or with a control Luciferase targeting shRNA (shLuc), before transfection or not with RNA:DNA hybrids for 3, 6, and 12 hours. Cells were harvested and analyzed for Ifnβ mRNA levels by RT-qPCR (Fig. 3A), as well as by WB against phosphorylated IRF3 (p1IRF3) (Fig. 3B). This showed that the absence of LysRS leads to up-regulated basal Ifnβ mRNA levels, which are further increased upon transfection of RNA:DNA hybrids. The kinetics of Ifnβ expression were faster in LysRS–knocked-down cells, suggesting that LysRS delays activation of the IFN response. These results were confirmed using the three prime repair exonuclease 1 (Trexl)–deficient MEF (MEF<sub>Trel1</sub>−/−), which is a well-characterized model of chronic inflammation that presents high levels of RNA:DNA hybrids together with elevated STING-dependent Ifnβ and interferon stimulated genes (ISGs) mRNA levels (fig. S3, A and B) (24). Following knockdown of LysRS in MEF<sub>Trel1</sub>−/−, we observed increased Ifnβ mRNA levels (Fig. 3C and fig. S3C) and increased levels of p1IRF3 both at steady state and upon RNA:DNA hybrid stimulation (Fig. 3D and fig. S3D). Conversely, overexpression of F/HA-LysRS, followed by RT-qPCR analysis showed decreased steady-state and RNA:DNA hybrid–induced Ifnβ mRNA levels (Fig. 3E), accompanied by decreased levels of
Fig. 3. The Lysyl tRNA synthetase inhibits RNA:DNA hybrid–induced IFN production. (A) Mean (±SEM) Ifnβ mRNA levels in shLysRS-expressing versus shLuc-expressing WT-MEF transfected or not with R:D for 3, 6, 9, and 12 hours. Representative graph (n = 4). (B) Whole-cell extracts from cells treated as in (A) were analyzed by WB using indicated antibodies. (C) Mean (±SEM) Ifnβ mRNA levels in shLysRS-expressing versus shLuc-expressing MEF TRE1−/− transduced or not with R:D for 6 hours (n = 7). (D) Whole-cell extracts from cells treated as in (C) were analyzed by WB using indicated antibodies. (E) Mean (±SEM) Ifnβ mRNA levels in MEF TRE1−/− overexpressing F/HA-LysRS following transfection or not with R:D for 6 hours (n = 4). (F) Whole-cell extracts from cells treated as in (E) were analyzed by WB using indicated antibodies. (G) Ifnβ mRNA levels in WT-MNF (mouse neonatal fibroblast) and MNF LysRS+/− (cells derived from two independent 1-day-old mice). Different colors indicate different mice. (H and I) WT-MNF and MNF LysRS+/− were infected with HSV-1 (H) or HSV-2 (I) at multiplicity of infection (MOI) = 1, and viral titers were measured 24 hours later. Data represent biological triplicates of cells derived from two independent 1-day-old mice. Different colors indicate different mice. (J and K) Mean (±SEM) Ifnβ mRNA levels in MEF TRE1−/− transduced with shLuc, shLysRS, shSting, or shLysRS and shSting prior (J) or after (K) transfection with R:D for 6 hours. Data are expressed relative to shLuc-expressing cells (n = 7). (L) WB analysis of whole-cell extracts from experiment performed as in (J) and (K). (M) Ifnβ mRNA levels in shLuc versus shLysRS-treated MEF TRE1−/− after transfection with R:D for 6 hours. (N) Ifnβ mRNA levels in shLuc versus shLysRS-treated MEF TRE1−/− after transfection with dsRNA (R:D) for 6 hours. (O) Ifnβ mRNA levels in shLuc versus shLysRS-treated MEF TRE1−/− after transfection with dsRNA (R:D) for 6 hours. Results in (M) to (O) are presented as mean Ifnβ mRNA levels from technical duplicates (representative experiments; n = 3). (C, E, I, J, and K) Unpaired t test, *P < 0.05, **P < 0.01, and ***P < 0.0001. All immunoblots show representative experiments. PFU, plaque-forming units.
pIFR3 (Fig. 3F). In further support of the role of LysRS in controlling innate immune responses, primary mouse neonatal fibroblasts heterozygous for LysRS (MNFs<sup>LysRS<sup>+/−</sup></sup>; fig. S3E) showed higher basal Ifnβ mRNA levels (Fig. 3G) than their wild-type counterparts (MNFs<sup>LysRS<sup>++</sup></sup>, together with elevated ISG mRNA levels, including Ifit1, Ifit2, 1sg15, and Cxcl10 (fig. S3F). Consistent with their ISG expression profile, MNFs<sup>LysRS<sup>−/−</sup></sup> poorly support herpes simplex virus (HSV) type 1 and 2 replication (Fig. 3, H and I). Thus, our data demonstrate that LysRS negatively regulates Ifnβ expression in a model of chronic inflammation and that the absence of LysRS leads to the establishment of an antiviral state.

Detection of RNA:DNA hybrids has been previously reported to lead to the activation of STING (Fig. 1A) (14). Thus, we next assessed whether LysRS-mediated inhibition of Ifnβ production may rely on inhibition of STING activation. To this aim, we knocked down both LysRS and Sting in MEF<sup>Trex<sup>−/−</sup></sup> and measured Ifnβ mRNA and pIFR3 levels by RT-qPCR and WB, respectively. This showed that while the absence of LysRS leads to increased Ifnβ mRNA levels at both steady state (Fig. 3J) and upon stimulation with RNA:DNA hybrids (Fig. 3K), such increase is lost in the absence of STING (Fig. 3, J and K). Consistently, knockdown of LysRS led to increased pIFR3 levels (Fig. 3L) that are lost in the double knockdown. We confirmed the dependency on STING by knocking down LysRS in WT-MEF before transfection with either RNA:DNA hybrids (Fig. 3M) and dsDNA (Fig. 3N), which are known to trigger STING-dependent signaling, or dsRNA (Fig. 3O), which signals through a different pathway (25). The absence of LysRS led to increased Ifnβ mRNA levels in response to RNA:DNA hybrids and dsDNA but not in response to dsRNA (Fig. 3, M to O). This is supported by increased levels of pIFR3 (fig. S3G). Thus, together, our data demonstrate that LysRS delays STING-dependent Ifnβ expression.

### LysRS delays the detection of RNA:DNA hybrids by cGAS

We next questioned the molecular mechanism through which LysRS inhibits STING activation. It was previously suggested that cGAS may bind to RNA:DNA hybrids because (i) in vitro RNA:DNA hybrids can stimulate cGAS-dependent cGAMP production (14) and (ii) RNA:DNA-dependent Ifnβ expression requires cGAS (Fig 1, A and B) (14). Given that we show that LysRS interacts with RNA:DNA hybrids (Fig. 2A), we hypothesize that LysRS may inhibit STING activation by preventing the association of cGAS with RNA:DNA hybrids. To test this hypothesis, we first monitored whether cGAS interacts with the RNA:DNA hybrids used in our assays. To this aim, T98G cells, which are cGAS deficient, were engineered to stably express FLAG-HA–tagged cGAS (F/HA-cGAS). The functionality of overexpressed cGAS was assessed by monitoring the phosphorylation status of IRF3 after transfection with RNA:DNA hybrids and dsDNA (fig. S4A). We prepared cytosolic and nuclear extracts (fig. S4B) according to (26) from cGAS overexpressing cells, and the cytosolic fraction was used for in vitro pull-down experiments using <sup>32</sup>P-RNA:DNA hybrids, <sup>32</sup>P-DNA, and ss<sup>32</sup>P-DNA immobilized on streptavidin affinity beads. Pulled-down material was assessed by WB, showing that cGAS binds RNA:DNA hybrids, albeit at lower levels than dsDNA. Next, we transfected F/HA-cGAS–expressing T98G cells with <sup>32</sup>P-RNA:DNA hybrids and ds<sup>32</sup>P-DNA before pull-down using streptavidin affinity beads. WB analysis of the pulled-down material showed that in cells, cGAS interacts both with dsDNA and with RNA:DNA hybrids (Fig. 4B). Thus, together, our data demonstrate that cGAS interacts with RNA:DNA hybrids.

Having established that both LysRS and cGAS can interact with RNA:DNA hybrids, we next wished to investigate the affinity of these interactions by calculating the dissociation constant (K<sub>d</sub>) of the interaction of cGAS or LysRS with RNA:DNA hybrids. To this end, we performed in vitro streptavidin pull-down assays using increasing amounts of recombinant proteins (fig. S4C) and a fixed amount of <sup>32</sup>P-RNA:DNA hybrids. Biotin was used as a mock ligand in this assay, at the lowest and highest doses of tested proteins. WB analysis (Fig. 4C) and quantification of the interaction (Fig. 4D) show a K<sub>D</sub> of ~8.05 nM for cGAS and of ~11.3 nM for LysRS. This indicates that LysRS and cGAS have similar affinity for RNA:DNA hybrids in vitro. Competition experiments showed that in vitro cGAS can compete with LysRS for association to RNA:DNA hybrids (Fig. 4E), while LysRS cannot replace cGAS (Fig. 4F).

Knowing that LysRS is an abundant protein and that cGAS is an ISG, their relative abundance in cells may be the key in their ability to compete. Therefore, to assess whether LysRS can interfere with the recruitment of cGAS to RNA:DNA hybrids, we measured intracellular cGAMP levels in the absence of LysRS, at steady state and upon RNA:DNA hybrid stimulation (Fig. 4G). This showed that knockdown of LysRS leads to higher levels of intracellular cGAMP both at steady state and upon stimulation with RNA:DNA hybrids, which correlate with higher levels of Ifnβ mRNA (Fig. 4H) and pIRF3 (Fig. 4I). Thus, our data indicate that LysRS interferes with cGAMP production in response to RNA:DNA hybrids, further implying that LysRS likely delays recognition of RNA:DNA hybrids by cGAS.

### Ap4A prevents STING-dependent Ifnβ expression

A role for LysRS in regulation of immune responses was previously suggested (15). In particular, under immunological stress, LysRS produces Ap<sub>4</sub>A, an evolutionarily conserved second messenger (27), that regulates both gene expression and immune responses (17, 18, 28). This led us to question whether the presence of RNA:DNA hybrids could be sensed by LysRS as a form of immunological stress and consequently stimulate the production of Ap<sub>4</sub>A, the latter potentially contributing to the inhibition of STING-mediated immune responses. To test this hypothesis, we transfected WT-MEF with RNA:DNA hybrids and measured intracellular Ap<sub>4</sub>A levels at 0, 3, 6, 9, and 12 hours after transfection (Fig. 5A). Activation of the cGAS-STING pathway was controlled by WB analysis of pIRF3 levels (Fig. 5B). This showed that RNA:DNA hybrids stimulate Ap<sub>4</sub>A production. Next, to assess whether RNA:DNA hybrid–dependent Ap<sub>4</sub>A production requires LysRS, we knocked down LysRS before transfection with RNA:DNA hybrids for 12 hours (Fig. 5, C and D). LysRS knockdown led to the expected decrease of intracellular Ap<sub>4</sub>A levels. In addition, the ability of RNA:DNA hybrids to stimulate Ap<sub>4</sub>A production is lost in LysRS knock-down cells, establishing RNA:DNA hybrids as trigger for LysRS-dependent Ap<sub>4</sub>A production.

To investigate whether Ap<sub>4</sub>A could have an impact on STING-dependent inflammation, we treated MEF<sup>Trex<sup>−/−</sup></sup> with Ap<sub>4</sub>A for 45, 90, and 180 min. We observed that Ap<sub>4</sub>A decreased Ifnβ mRNA levels over the three-hour time course (Fig. 5E). In addition, simultaneously treating WT (WT-MEF) and STING knockout MEFs (MEF<sup>Sting<sup>−/−</sup></sup>) with DMXAA and Ap<sub>4</sub>A showed that, while cotreatment reduced the ability of DMXAA to induce Ifnβ expression in WT-MEF by half (Fig. 5F), no decrease was observed in MEF<sup>Sting<sup>−/−</sup></sup> (Fig. 5G). Because it is reported that Ap<sub>4</sub>A has poor capacity to cross the plasma membrane (29) and is quickly catabolized in cells (30), we next tested the ability of JB419 [nonhydrolyzable analog of Ap<sub>4</sub>A (31)] to modulate Ifnβ
Fig. 4. LysRS delays the detection of RNA:DNA hybrids by cGAS. (A) In vitro interaction. Left: Experimental scheme. T98G cells stably expressing F/HA-cGAS (T98G-F/HA-cGAS) were fractionated. The obtained cytosolic fraction was incubated with either 20 pmol of 9RD, 9OD, or ssDNA before streptavidin affinity pull-down. Right: Input and eluates were immunoblotted with anti-HA antibody to detect F/HA-cGAS and the indicated antibodies. (B) In cell interaction. Left: Experimental scheme. T98G-F/HA-cGAS cells were transfected or not with 9RD, and 9OD. Six hours after transfection, cells were harvested, and whole-cell extracts were prepared and used for streptavidin affinity pull-down. Right: Inputs and eluates were immunoblotted using anti-HA antibody. (C) In vitro streptavidin pull-downs were performed using 20 pmol of 9RD and increasing amounts of recombinant proteins (2.5, 5, 10, 20, 40, 80, and 160 nM). Biotin (20 pmol) was used as mock ligand at the lowest and highest doses of recombinant proteins. Input and eluates were immunoblotted with anti-GST antibody. (D) Signals from WB in (C) were quantified by ImageJ and plotted in Prism, and data were fitted by the nonlinear regression method for one-site specific binding. x axis: amounts (nM) of recombinant protein and y axis: absolute binding expressed as arbitrary units (AU). Representative graph (n = 4). (E and F) Competition experiments. Streptavidin-immobilized 9RD were incubated with 10 nM LysRS and increasing doses of cGAS (2.5, 5, 10, 20, 40, 80, and 160 nM) (F) and with 10 nM cGAS and increasing doses of LysRS (2.5, 5, 10, 20, 40, 80, and 160 nM) (E). (H) LysRS was knocked down in MEF^TREX1^ cells before transfection with RD for 3, 6, and 9 hours. Cells were harvested, and cell pellets were processed for intracellular cGAMP measurements using the cGAMP ELISA Kit (CISBIO). Graph represents mean ± SD from technical duplicates of pg cGAMP per 10^6 cells. Representative graph (n = 3). (I) Ifnβ mRNA levels, measured in cells treated as in (G), are expressed as mean ± SEM from technical duplicates. Data are expressed relative to shLuc-expressing cells. Representative graph (n = 3). (B) Whole-cell extracts from experiment presented in (G) were analyzed by WB using the indicated antibodies. All immunoblots show representative experiments.
Fig. 5. Ap4A inhibits STING-dependent Ifnβ expression. (A) Mean (±SEM) Ap4A levels in WT-MEFs transfected or not with R:D for 3, 6, 9, or 12 hours are expressed as pmol of Ap4A per 10^6 cells (n = 3). (B) Whole-cell extracts from cells treated as in (A) were analyzed by WB using the indicated antibodies. (C) Mean (±SEM) Ap4A levels in HeLa cells stably expressing shLuc or shLysRS transfected or not with R:D hybrids for 12 hours are expressed as pmol of Ap4A per 10^6 cells (n = 5). (D) Whole-cell extracts from cells treated as (C) were analyzed by WB using the indicated antibodies. (E) Mean (±SEM) Ifnβ mRNA levels in untreated MEF Trex1−/− versus MEF Trex1−/− treated with 500 μM Ap4A for 45, 90, or 180 min (n = 6). (F) Mean (±SEM) Ifnβ mRNA levels in WT-MEF stimulated with 200 μM DMXAA and treated, or not, with 500 μM Ap4A for 2 hours (n = 13). Data are normalized to nontreated control. (G) Mean (±SEM) Ifnβ mRNA levels in MEF Trex1−/−→ R:D stimulated with 200 μM DMXAA and treated, or not, with 500 μM Ap4A for 2 hours (n = 3). Data are normalized to nontreated control. (H) Mean (±SEM) Ifnβ mRNA levels in MEF Trex1−/−→ R:D treated with 500 μM Ap4A or 500 μM JB419 for 3 hours versus untreated MEF Trex1−/− (n = 4). (I) The 3D modeling of docked cGAMP (left) or Ap4A (right) in the cleft of STING obtained using Amber force field and the crystal structure of human STING complexed to cGAMP (PDB: 4KSY). Ap4A has adopted a horseshoe-like conformation. (J) Predicted molecular interactions that stabilize Ap4A in the active site of STING. Atoms are pink spheres (acidic, red outline; basic, blue outline). Interactions are depicted by arrows showing the direction of the electron exchange (donor or acceptor), with backbone interactions in blue and sidechain interactions in green. The dotted contour defines the proximity of the available conformational space for the depicted compound. (K) WB analysis of in vitro binding experiment using 100 μg of GST, GST-HINT or GST-STING139–378, and 10 μM streptavidin-immobilized biotinylated Ap4A. (L) WB analysis of in vitro binding experiment performed using 100 μg of GST or GST-STING139–378 and 10 μM streptavidin-immobilized biotinylated Ap4A. Competition was performed with 100 μM cGAMP, DMXAA, JB419, or Ap4A as indicated. (M) Proposed model. At steady state (left), LysRS interacts with R:D, delaying the recruitment of cGAS to R:D. LysRS interaction with R:D also stimulates Ap4A production, which buffers STING activation. In the absence of LysRS (right), cGAS detects R:D, producing cGAMP and resulting in STING activation. IFN is produced, inducing the establishment of a positive feedback loop, which increases the total amount of cGAS and additional ISGs. (A, C, E, F, G, and H) Unpaired t test; ns, nonsignificant; *P < 0.05, **P < 0.01, and ***P < 0.0001. All immunoblots show representative experiments.

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expression. We observed that treatment of MEFs with JB419 leads to decreased Ifnβ mRNA levels, comparable to those observed upon treatment with Ap4A (Fig. 5H). These experiments suggest that Ap4A and its stable JB419 nonhydrolyzable analog can decrease Ifnβ mRNA levels in a model of STING-dependent inflammation. In addition, Fig. 5G suggests that Ap4A acts through STING, opening the possibility that Ap4A may interact with STING. Therefore, we next questioned whether Ap4A acts through STING to delay the type I IFN response by binding to the cGAMP binding pocket of STING. To this aim, we used Amber force field (32) and the previously published cocrystal of STING with cGAMP (Protein Data Bank (PDB): 4KSY) (4) to compare the binding of Ap4A and cGAMP to STING. This predicted that cGAMP and Ap4A adopt a similar three-dimensional (3D) conformation upon docking to STING (Fig. 5I and fig. S5A). Comparison of the interactions between the in silico–docked Ap4A to STING (Fig. 5I) to those in the crystal structure of STING and cGAMP (fig. SSB) indicates that similar interactions are established. Comparison of the potential energies indicates that the Ap4A-STING complex is more dynamic than the cGAMP-STING complex and that the interaction of Ap4A with STING is thermodynamically less stable (table S1). This was confirmed by molecular dynamics simulations showing that the Ap4A-STING complex is highly unstable compared to cGAMP-STING (fig. S5C).

On the basis of these predictions, we performed in vitro binding assays using recombinant GST-tagged C-terminal binding domain of mouse STING (GST-STING139–378; fig. S5D) and streptavidin-immobilized biotinylated Ap4A. GST and GST-tagged mouse Histidine triad nucleotide-binding protein 1 (Hint1) (fig. S5D), reported to bind Ap4A (33), were included as negative and positive controls, respectively. We observed that, as predicted in silico, STING139–378 interacts with Ap4A (Fig. 5K). Also in agreement with in silico predictions (table S1), competition experiments using unlabeled Ap4A, cGAMP, or DMXAA as competitors showed that binding of biotinylated Ap4A to STING can be easily displaced (Fig. 5L). The JB419 nonhydrolyzable analog of Ap4A also competed for the binding of STING to Ap4A (Fig. 5L), further suggesting that JB419 also interacts with STING. Thus, these data indicate that Ap4A can bind to STING. Our observations also confirm the in silico predictions that Ap4A and cGAMP share the same binding pocket on STING and that the binding of Ap4A to STING is less stable as compared to that of cGAMP. We therefore propose a model where LysRS and Ap4A regulate the activation of STING by two complementary mechanisms. First, LysRS recognizes cytosolic RNA:DNA hybrids, delaying their detection by cGAS. Second, binding of LysRS to RNA:DNA hybrids stimulates the production of Ap4A, which delays the binding of cGAMP to STING, resulting in delayed IFN response (Fig. 5M, left). In the absence of LysRS, cGAS recognizes RNA:DNA hybrids leading to a higher cGAMP production and subsequent type I IFN response (Fig. 5M, right).

**LysRS-mediated negative regulation of Ifnβ expression is conserved in zebrafish.**

Last, we wished to challenge our finding in vivo in zebrafish embryos, taking advantage of the close evolutionary resemblance of their innate antiviral pathways to that of humans (34). We first verified that recombinant zebrafish LysRS (zlLysRS) interacts with RNA:DNA hybrids in vitro (Fig. 6A). We then performed whole-body knockdown of the unique zebrafish ortholog of LysRS (zlLysRS) by injecting wild-type one-cell zebrafish embryos with a splice-blocking morpholino targeting zlysrs (Fig. 6B) or with a control morpholino. Microscopy analysis of fish morphology showed no developmental defect (Fig. 6C). Quantification of the expression levels of type I IFN (ifnβ) and the IFN-response gene isg15 at 72 hours after injection showed that knocking down lysrs led to a global up-regulation of the IFN response (Fig. 6D). This establishes that negative regulation of type I IFN by LysRS is conserved in fish and mammals.

**DISCUSSION**

In summary, we show that RNA:DNA hybrids are bona fide ligands of cGAS and that LysRS represses RNA:DNA hybrid–dependent cGAS-STING activation through two complementary mechanisms. First, LysRS interacts with RNA:DNA hybrids, preventing, or delaying, their recognition by cGAS. Consistently, knocking down LysRS leads to enhanced cGAMP production, attesting to increased cGAS activation. This infers that in the absence of LysRS the bioavailability of RNA:DNA hybrids for recognition by cGAS is enhanced. Considering that cGAS is an ISG and that LysRS is highly expressed in cells, their relative abundance is likely to be a key determinant of STING activation. In pathological situations where acute nucleic acid–induced STING stimulation occurs, such as upon chemotherapy or radiotherapy regimens or viral infections (35), it is likely that...
masking of RNA:DNA hybrids by LysRS is critical. Second, we identify here that simulation with RNA:DNA hybrids leads to LysRS-dependent Ap4A production that, in turn, delays the activation of type I IFN responses by binding to the cGAMP binding pocket of STING, confirming its signaling function in mammalian cells (17, 18, 28).

LysRS-dependent inhibition of STING is likely complementary to existing cellular processes that regulate cytosolic levels of nucleic acids to avoid unwanted triggering of the cGAS-STING pathway (7). Enzymes such as deoxyribonuclease II (DNase II), TREX 1, RNaseH 1, or RNaseH 2 are in charge of degrading cytosolic nucleic acids, preventing their detection. Thus, at resting state, despite the potential generation of RNA:DNA hybrids through processes such as transcription-replication collision or immunoglobulin class switching (11), it is likely that LysRS is mostly engaged in translation. Hence, in the absence of such nucleases, the LysRS-Ap4A pathway may play a pivotal role. For example, it is reported that the absence of RNaseH2 leads to up-regulation of STING-dependent type I IFN responses through the appearance of dsDNA species containing ribonucleotides that induce DNA damage rather than through an increase of total RNA:DNA hybrids (36). While it can be expected that, in this model, LysRS would not have its inhibitory effect, due to the lack of RNA:DNA hybrids, it would be important to assess the impact of treatment with Ap4A or JB419. In addition, it has been shown that members of the MSC can interfere with retroviral life cycles. In particular, LysRS interferes with the HIV-1 replication cycle (15) and that HIV-1 infection promotes nuclear translocation of LysRS (37), a process that may lead to the production of Ap4A (18), favoring the viral life cycle by decreasing the antiviral response. It would be therefore important to test whether LysRS is able to bind HIV-1-derived RNA:DNA hybrids, whether this leads to Ap4A production, and the impact of the latter on the viral life cycle.

While our work reveals that the LysRS-Ap4A axis is a key pathway involved in physiological regulation of STING activation, it also raises questions regarding the involvement of the MSC in regulating the innate immune responses. Other members of the MSC, such as bifunctional glutamate/proline-tRNA ligase (EPRS) (38), have already been shown to play roles in the regulation of immune responses. Thus, it would be important to examine whether knocking down additional members of the MSC, together with LysRS, can enhance the effect of LysRS on the cGAS-STING pathway. In addition, we also show that LysRS-mediated inhibition of type I IFN response is a conserved mechanism in mammals and fish. However, it is likely that the overall involved molecular mechanisms differ. While it has been described that in vitro, the zebrafish orthologs of cGAS can produce cGAMP (39), it has been previously reported that cGAS is dispensable for innate immune detection, to the contrary of probable ATP-dependent RNA helicase DDX41 and ATP-dependent RNA helicase A (DHX9), in zebrafish (40). It would therefore be important to test whether LysRS-mediated inhibition of IFN responses may be mediated by sequestration of RNA:DNA hybrids from other sensors than cGAS.

Last, our data also show that the JB419 cell-permenant nonhydrolyzable analog of Ap4A (31) antagonizes STING to levels similar to those observed for Ap4A. Thus, this compound bears the potential for translation to the clinic and opens the way to the design of derived compounds with higher affinity for STING or increased retention time in the cGAMP-binding pocket. While such compounds would have to be carefully assessed for side effects and cytotoxicity before testing in animal models of chronic inflammation, our study provides basis for the design of a novel class of pharmaceutical inhibitors of STING. In addition, activating endogenous LysRS to produce Ap4A may also be a relevant approach to study the effect of long-term exposure to these compounds on global immune responses. Our work thus warrants further studies to better characterize the role of the LysRS-Ap4A axis in human pathologies where inflammatory responses are reported.

MATERIALS AND METHODS

Study design

The present study aimed at identifying the molecular mechanisms governing regulation of STING activation. Experiments performed in vitro, in cells, and in vivo were carefully controlled. In vitro experiments performed using recombinant proteins or cell lysates were performed independently using different protein preparations. Experiments performed in cells were performed in technical duplicates or triplicates, and biological repetitions are indicated in the figure legends. In vivo experiments in zebrafish were repeated twice on pools of three individual larvae. Morpholino injections were blinded. No outlier was excluded in the course of the analyses.

Cells and cell cultures

293T, A549, HeLa, HeLa-S3, WT-MEF, MEFSting−/−, MEFcGas−/−, MEFTrex1−/−, T98G, and T98GFHA-cGas were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% glucose. T98G overexpressing F/HA-cGas were generated by transducing parental T98G with viral particles produced by using the pOZ-F/HAcGAS construct and selected with puromycin. Cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% glucose. A549 were obtained from S. Goodborn. WT-MEF, MEFSting−/−, and MEFcGas−/− were a gift of S. R. Paludan. MEFTrex1−/− were obtained from J. Rehwinkel, and parental T98G were obtained from C. Goujon.

Mouse neonatal fibroblast

MNFs were purified as described by the Krishna Halai laboratory. Briefly, skin was isolated from newborns at day 1, incubated in Krebs-Ringer Hepes (KRH) buffer containing 10 mM Hepes and 1% penicillin/streptomycin and minced in pieces with a scalpel before digestion in KRH/ATB buffer supplemented with collagenase (1 mg/ml) at 37°C. After centrifugation at 1000 rpm for 5 min, the supernatant was discarded, and the isolated cells put in culture in DMEM, 20% FBS, and 1% penicillin/streptomycin.

Plasmids

shRNAs targeting LysRS and STING were cloned into the pSUPERIOR retro.puro vector from Oligoengine, according to the manufacturer’s instructions. N-terminally Flag- and HA-tagged codon-optimized mouse LysRS (MWG Eurofins) was cloned into the pOZ retroviral vector. For bacterial expression of recombinant proteins, the human LysRS gene was amplified by PCR from complementary DNA (cDNA) of HeLa cells. The cDNA and codon-optimized human cGas (MWG Eurofins) were cloned into the pGEX-4T1 plasmid. Codon-optimized mouse LysRS was cloned into the pGEK-4T1 plasmid. Danio rerio LysRS (zLysRS) was amplified by PCR from the cDNA of zebrafish larvae and cloned in pGEX-4T1.
Viral particle production and infection

shRNA-expressing retroviral particles were produced by cotransfection of 2 × 10^6 293T cells with 5 µg of shRNA-containing pSUPERIOR, 2.5 µg of murine leukemia virus (MLV) GagPol, and 2.5 µg of A-MLV envelope, using the standard calcium phosphate transfection protocol. Retroviral particles containing the transgene encoding Flag- and HA-tagged LysRS (F/HA-LysRS) were produced following the same procedure except that cells were cotransfected with 5 µg of pOZ-F/ HALysRS, 2.5 µg of MLV GagPol, and 2.5 µg of A-MLV envelope. Viral particles were harvested 48 hours after transfection, filtered with 0.45 µm filters, and used for transduction.

For knockdown of LysRS, 10^5 cells were seeded 24 hours before transduction. Medium was replaced 10 hours after transduction, and transfection was performed 72 hours later. A similar procedure was used for knockdown of LysRS and STING, except that retroviral particles containing shRNAs targeting LysRS and STING were added at the same time. For expression of F/HA-LysRS, 5 × 10^5 cells were seeded 24 hours before transduction. Twenty-four hours later, viral particles were added, and medium was replaced after 10 hours. RNA:DNA hybrids were transfected 96 hours later.

HSV plaque assay

MNFs were seeded at a density of 0.5 to 1 × 10^5 cells per well in a 24-well plate (Nunc) in 500 µl of DMEM + 1% FBS 24 hours before infection with HSV-1 KOS or HSV-2 333 at multiplicity of infection (MOI) of 0.1. Supernatants were harvested 24 hours later, and virus yield was quantified using the plaque titration assay on Vero cells.

Protein purification

Recombinant GST-HINT, GST-LysRS, GST-cGAS, and GST-STING (amino acids 139 to 378), and GST-ZflLysRS were produced in *Escherichia coli* BL21 cells. *E. coli* transformed with GST or GST expression constructs were grown in LB medium at 37°C to an absorbance at 600 nm of 0.6 before induction with 0.25 mM isopropyl-β-D-1-thiogalactopyranoside overnight at 16°C. Bacteria were harvested by centrifugation, resuspended in TETN-100 buffer (50 mM tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100, supplemented with lysozyme (2 mg/ml; Sigma), 10 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), and incubated on ice for 30 min. Salt and detergent concentrations were increased, respectively, to 400 mM and 0.5% before sonication. Lysates were resuspended in TETN-400 buffer (50 mM tris-HCl (pH 8), 400 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100, supplemented with 10 mM β-mercaptoethanol and 0.5 mM PMSF) and were clarified by centrifugation at 13,000 rpm for 30 min at 4°C before incubation with the appropriate volume of glutathione-Sepharose beads for 4 hours at 4°C. Sepharose beads were washed three times with ice-cold TETN-400, and recombinant proteins were eluted with elution buffer (150 mM NaCl and 50 mM tris-HCl (pH 8)) supplemented with 30 mM reduced γ-glutathione. Eluates were quantified by Coomassie staining.

Nuclear cytoplasmic fractionation

T98G*F/HA-LysRS* cells were fractionated according to Gentili *et al.* (26) Briefly, cells were harvested and cell pellet was washed once in 400 µl of cold cytoplasmic lysis (CL) buffer [10 mM Heps (pH 7.9), 10 mM KCl, and 1.5 mM MgCl2] in the presence of 0.5 mM PMSF and centrifuged at 300g for 4 min. The supernatant was discarded, and cell pellet was lysed by adding three cell pellet volumes of cold CL for 10 min on ice, with gentle flicking. Cytosolic extract was obtained by adding 0.625% final of NP-40 for an additional of 5 min on ice, with gentle flicking. Nuclei were pelleted at 16,000g for 5 min, and cytosolic extracts were collected in a fresh tube. Nuclei were then lysed by adding three cell pellet volumes of cold nuclear lysis buffer [420 mM NaCl, 20 mM Heps (pH 7.9), 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, and 0.5 mM PMSF] for 15 min on ice, with gentle flicking. Nuclear lysates were vortexed and sonicated for 20 min at 4°C in a Bioruptor ultrasonic bath. Nuclear lysates were cleared by centrifugation at 16000g for 5 min.

Biotinylated nucleic acid pull-down using recombinant protein

In vitro interaction between RNA:DNA hybrids and recombinant proteins was performed using Dynabeads M280. Beads were blocked overnight in blocking buffer [20 mM Heps (pH 7.9), 15% glycerol, 0.05% NP-40, and 50 mM NaCl, supplemented with 2 mM dithiothreitol (DTT), 100 mM NaCl, and bovine serum albumin (BSA; 10 mg/ml)]. After three washes in 1x wash buffer [5 mM tris-HCl (pH 7.5), 1 mM EDTA, and 1 M NaCl], M280 beads were coupled to nucleic acids according to the manufacturer’s instructions. Briefly, beads were incubated in 50 µl of 2x wash buffer [10 mM tris-HCl (pH 7.5), 2 mM EDTA, and 2 M NaCl] containing 20 pmol of RNA:DNA hybrids at 25°C for 15 min. Protein binding was performed at 37°C for 30 min with increasing amounts of recombinant proteins in 20 mM Heps (pH 7.9), 15% glycerol, 0.05% NP-40, and 150 mM NaCl, supplemented with 2 mM DTT, 2 mM PMSF, and RNase inhibitor. When indicated, hybrids were treated with 30 U of RNaseH (Ambion) in RNaseH buffer [50 mM tris-HCl (pH 8), BSA (50 mg/ml), 1 mM DTT, and 4 mM MgCl2] for 1 hour at 30°C, before binding to the beads. After three washes, bound material was eluted in 30 µl of Laemmlı buffer. Pull-down in competition between hcGAS and hLysRS was performed as described above except that the two proteins were added at the same time to the beads coupled to the RNA:DNA hybrids.

Biotinylated nucleic acid pull-down using cell extracts

Interaction of endogenous protein and transfected biotinylated nucleic acids was tested by transfecting HeLa cells with nucleic acids (2 µg/ml) using JetPrime according to the manufacturer’s protocol. Six hours after transfection, cells were harvested and lysed in TENTG-150 [20 mM tris-HCl (pH 7.4), 0.5 mM EDTA, 150 mM NaCl, 10 mM KCl, 0.5% Triton X-100, 1.5 mM MgCl2, and 10% glycerol, supplemented with 10 mM β-mercaptoethanol and 0.5 mM PMSF] on ice for 30 min. Lysates were centrifuged at 13,000 rpm for 30 min at 4°C. Equal amounts of whole-cell lysates were incubated for 3 hours at 4°C with a wheel with 3 µg (30 µl) Dynabeads M280 blocked overnight at 4°C as described above. After three washes in 20 mM tris-HCl (pH 7.4), 0.5 mM EDTA, 0.05% Triton, 0.1% Tween, 150 mM NaCl, 10% glycerol, and 5 mM MgCl2, bound material was eluted in Laemmlı buffer.

In vitro biotinylated nucleic acid pull-down

Pull-down was carried out using 3 µg (30 µl) of Dynabeads M280 per condition. Beads were blocked overnight as described above. After three washes in 1x wash buffer, 100 pmol of nucleic acid was coupled to M280 beads according to the manufacturer’s instructions before equilibration in dialysis buffer [20 mM tris-HCl (pH 7.4), 0.5 mM EDTA, 150 mM NaCl, 10% glycerol, and 1.5 mM MgCl2]. Cytosolic extracts from HeLa S3 cells, prepared using the Dignam protocol, were...
diluted in dialysis buffer supplemented with 2% Tween, 1% Triton, 10 mM β-mercaptoethanol, and 0.5 mM PMSF and centrifuged at 13,000 rpm for 30 min at 4°C. One milliliter of diluted lysate was added to the beads and incubated at 4°C on a rocker for 4 hours in low-binding tubes (Axygen). Three consecutive washes were performed in 20 mM tris-HCl (pH 7.4), 0.5 mM EDTA, 0.05% Triton, 0.1% Tween, 150 mM NaCl, 10% glycerol, and 5 mM MgCl₂. Tubes were changed at first and last washes. Bound material was eluted in 30 μl of Laemmli buffer.

**Pull-down using biotinylated Ap₄A and recombinant proteins**

In vitro interaction between STING and biotinylated Ap₄A was tested using Dynabeads M280, blocked as described above. Ten micromolar of biotinylated Ap₄A (BioLog) or biotin was bound to the beads at 25°C for 30 min in 2× wash buffer. Thirty, 50, or 100 μg of recombinant proteins (mSTING, mHINT, and GST) was incubated with the beads for 1 hour at 4°C on the wheel in a final volume of 200 μl in 150 mM NaCl, 50 mM tris-HCl (pH 8.0), 10 mM MgCl₂, BSA (0.1 μg/μl), 10 mM β-mercaptoethanol, and 0.5 mM PMSF. Competition was performed after protein binding using a 10-fold excess of the competitor (DMXAA, 2′3′-cGAMP, Ap₄A, or JB419) for 1 hour at 4°C on wheel in a final volume of 200 μl in the buffer described above. After three washes in 150 mM NaCl, 50 mM tris-HCl (pH 8.0), 10 mM MgCl₂, 0.1% Tween, BSA (0.1 μg/μl), 10 mM β-mercaptoethanol, and 0.5 mM PMSF, bound material was eluted in 30 μl of Laemmli buffer.

**S9.6 immunoprecipitation**

Endogenous RNA:DNA hybrids were immunoprecipitated using the S9.6 antibody. Briefly, HeLa cells were crosslinked with 1% paraformaldehyde (PFA) for 20 min at room temperature. PFA was quenched by incubation with 125 mM glycine for 5 min at room temperature. Cells were harvested and lysed in TENTG-150 on ice for 30 min. Lysates were centrifuged at 13,000 rpm at 4°C for 30 min. The soluble fraction was precleared for 20 min at 4°C with 15 μl of agarose protein G beads, followed by incubation overnight at 4°C with 30 μl of agarose protein G beads coupled to 10 μg/ml of either irrelevant mouse immunoglobulin G (IgG) or S9.6 antibody. Beads were washed five times in TENTG-150 buffer, and immunoprecipitated material was eluted in Laemmli buffer. For phosphorylated protein analysis, buffer was supplemented with PhosphoSTOP (Roche) before whole-cell extraction. Protein quantification was performed using Bradford assay. Samples were resolved on SDS-PAGE, and proteins were transferred onto nitrocellulose membranes. Primary antibodies used include anti-phospho IRF3 (1:500; Cell Signaling 4D4G; 1:500; Abcam Ab76493), anti-IRF3 (1:1000; Cell Signaling D614C), anti-phospho TBK1 (1:1000; Cell Signaling D52C2), anti-TBK1 (1:1000; Cell Signaling D1B4), anti-STING (1:1000; Cell Signaling D2P2F), anti–HA (1:10000; Roche), anti–glyceraldehyde-phosphate dehydrogenase (GAPDH; 1:5000; Proteintech Europe 800004-1-Ig), anti-AMP1 (1:1000; Bethyl Laboratories A304896A-M), anti-DARS (1:1000; Bethyl Laboratories A304799A-M), anti-human LysRS (1:1000; Bethyl Laboratories A300630A-M), anti-mouse LysRS (1:2000; Proteintech Europe 14951-1AP), anti-GST (1:10000; Bethyl Laboratories A190122A), anti PARP-1 (1:500; Santa Cruz Biotechnology F-2 sc8007), anti-tubulin (1:2000; Proteintech Europe 66031-1-Ig), anti–TREX-1 (1:250; Santa Cruz Biotechnology C-11 sc133112), and anti-cGAS mouse specific (1:1000; Cell Signaling D3080). The S9.6 antibody was a gift from S. Leppla. All secondary antibodies (Cell Signaling) were used at 1:2000 dilution. Signal was visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific), and images were acquired on a ChemiDoc (Bio-Rad).

**Morpholino knock-down experiments**

Eggs of wild-type AB zebrafish (one-cell stage) were microinjected with 4 ng of the KARS-specific antisense morpholino (MO3i3, TC-CATATTCGGTACTCATGTACA) or with a control morpholino (M0ctl, GAAAGCATGGCATCTGGATCATCGA). The KARS-specific MO targets the splice donor site between exon 3 and intron 4. Efficiency of knockdown was assessed by RT-qPCR with splice-sensitive primers [TGGACCCCAATCAATACTTCAAG (forward) and GGTCTCCAGGCTGAAGGTGGTTAT (reverse)]. Embryos then developed with no obvious morphological defects at 28°C. At 72 hours after injection, embryos were collected for gene expression analysis by RT-qPCR.

**Molecular modeling**

Molecular docking of Ap₄A on STING was performed using ZDOCK version 3.0 using the STING crystal structure (PDB: 4KSY). RDOCK was used to refine and quickly evaluate the results obtained by ZDOCK. Energy minimization was performed to remove the geometrical strain from the top-ranking poses of Ap₄A during the docking experiments. Top hit complexes of Ap₄A and STING were subjected to extensive energy minimization run using the Amber99 force field as it is implemented into Gromacs, version 4.5.5, via the Gromita graphical interface, version 1.07. An implicit generalized born solvation was chosen at this stage, in an attempt to speed up the energy minimization process.

The interaction space and binding potential of each docking conformation were further explored by subjecting the molecular complexes to unrestrained molecular dynamics simulations using the Gromacs suite, version 4.5.5. Molecular dynamics took place in a periodic environment, which was subsequently solvated with phosphatidylcholine membrane using the truncated octahedron box extending to 7 Å from each molecule. Partial charges were applied, and the molecular systems were neutralized with counter ions as required. The temperature was set to 300 K, and the step size was
set to 2 fs. The total run of each molecular complex was 100 ns, using the NVT ensemble in a canonical environment. NVT stands for number of atoms, volume, and temperature that remain constant throughout the calculation. The results of the molecular dynamics simulations were collected into a molecular trajectory database for further analysis.

**Determination of intracellular Ap4A levels in MEF and HeLa**

Cellular nucleotide extraction and Ap4A determination were performed using a sensitive luminescence-based assay. Briefly, nucleotides were extracted from cells by adding 5 ml of ice-cold chloroformic acid and subsequent neutralization with an equal volume of 0.6 M tri-octylamine in 1,1,2-trichlorotrifluoroethane. Following centrifugation at 500g for 5 min, the top aqueous layer was removed, and 10 U of thermo-sensitive alkaline phosphatase (Thermo Fisher Scientific) was added to degrade adenosine triphosphate (ATP). Remaining nucleotides were concentrated using DEAE-Sephacel beads. Following mixing and centrifugation at 12,000g for 1 min, beads were washed with water. Nucleotides bound to DEAE-Sephacel were eluted twice with 1 M triethylammonium bicarbonate (pH 8.5), and eluates were vacuum-dried. Nucleotides from the two eluates were combined and dissolved in 120 µl of Ap4A assay buffer [25 mM Hepes–NaOH (pH 7.8) and 5 mM magnesium acetate]. To remove any remaining ATP, a further 10 U of alkaline phosphatase was added, incubated at 37°C, and subsequently incubated at 95°C to denature alkaline phosphatase. Ap4A levels in 10 µl of sample were determined by adding 50 µl of Bacter GLO (Promega). Background ATP levels were first measured on a Berthold Lumat 9507, and once stabilized, recombinant Ap4A hydrolase was added to cleave Ap4A (ATP + AMP), and levels of ATP were measured by the increase in luminescence. Levels of Ap4A in samples were determined by comparison to standards and expressed as pmol per 10^6 cells.

**cGAMP ELISA**

cGAMP enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer’s protocol using the Cayman Chemical 2′3′-cGAMP ELISA Kit (Bertin Bioreagents). MEF Trex−/− were seeded at 3 × 10^6 in 150-cm² plates 24 hours before transfection. Forty-eight hours later, cells were seeded at 3 × 10^6, and once stabilized, recombinant Ap4A hydrolase was added to cleave Ap4A (ATP + AMP), and levels of ATP were measured by the increase in luminescence. Levels of Ap4A in samples were determined by comparison to standards and expressed as pmol per 10^6 cells.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 7. Unpaired or one-column t test was performed as indicated in figure legends.

**Compounds (PubChem CID)**

DMXAA (Vadimezan): 123964
Ap4A (Diadenosine tetraphosphate): 21706
2′3′ cGAMP (cyclic guanosine monophosphate-adenosine monophosphate): 135564529

**Oligonucleotide sequences**

mIFNβ fw 5′-CTGGGTTCCTCTGCTGCT TCTCCA-3′
mIFNβ rv 5′-TTTCTCGCTCATCTCCATA GGGATC-3′
mSTING fw 5′-TTGGTTACTTTGGGTTGATCTTT-3′
mSTING rv 5′-GCACACTGAGCATGTT GTTATG-3′
mLysRS fw 5′-GTACTGCCCTGGAATAC GGGG-3′
mLysRS rv 5′-TTCACGTGCGTTGTCGAG TT-3′
mGAPDH fw 5′-TTCAACACCATGGAGAAC GGC-3′
mGAPDH rv 5′-GGCATCGACTCGTGGCATGTA-3′
zsACTIN fw 5′-CGAGCGTCTTCTCCCATCA 3′
zfsACTIN rv 5′-TCACACATGGAGAAC GGC-3′
zsIFNβ fw 5′-GAATGGCTTGGCCGATACAGGATA-3′
zfsIFNβ rv 5′-CGAGCGTCTTCTCCCATCA 3′
zfsLysRS fw P2 5′-TGACCACCAATCAATCTTCAAG-3′
zfsLysRS rv P2 5′-GGTCTCCAGGCTGAGTTGTAT 3′
Oligo sequences for annealing
RNA 2 or RNA 2 were annealed with RNA 5 to generate non-biotinylated or biotinylated RNA: DNA hybrids; DNA 9 or DNA 9 were annealed with DNA 6 to obtain non-biotinylated or biotinylated dsDNA. Annealing was performed in a MasterCycler nexus gradient (Eppendorf) in an annealing buffer [60 mM NaCl, 5 mM tris–HCl (pH 7.5), and 0.2 mM EDTA] using the following program: 4 min at 95°, 85°, 82°, 80°, 78°, 75°, and 72°C, followed by 10 min at 70°C and slow cooling down to 10°C over a period of 2 hours. The quality of annealed material was controlled by running 500 ng of annealed material on a 10% nondenaturing acrylamide gel using ssRNA and ssDNA as controls.

RNA 2: UUU CAA UUC CUU UUA GGA UUA AUC UUG AAG AAU GAG AA A
RNA 5: UUA ACU CUA UCU AGA AAA UUC CUU UUA GGA AUU GGA AUU AA
DNA 9: TTA ACT CTG TTA AGA TTA ATC TTA AAA

SUPPLEMENTARY MATERIALS
Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/21/eaax3333/DC1

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