SLC19A1 transports immunoreactive cyclic dinucleotides

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The accumulation of DNA in the cytosol serves as a key immunostimulatory signal associated with infections, cancer and genomic damage1–4. Cytosolic DNA triggers immune responses by activating the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway5. The binding of DNA to cGAS activates its enzymatic activity, leading to the synthesis of cyclic GMP–AMP (cGAMP)6–7. This cyclic dinucleotide (CDN) activates STING6, which in turn activates the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), promoting the transcription of genes encoding type I interferons and other cytokines and mediators that stimulate a broader immune response. Exogenous 2′,3′-cGAMP produced by malignant cells9 and other CDNs, including those produced by bacteria10,12 and synthetic CDNs used in cancer immunotherapy13,14, must traverse the cell membrane to activate STING in target cells. How these charged CDNs pass through the lipid bilayer is unknown. Here we used a genome-wide CRISPR-interference screen to identify the reduced folate carrier SLC19A1, a folate–organic phosphate antiporter, as the major transporter of CDNs. Depleting SLC19A1 in human cells inhibits CDN uptake and functional responses, and overexpressing SLC19A1 increases both uptake and functional responses. In human cell lines and primary cells ex vivo, CDN uptake is inhibited by folates as well as two medications approved for treatment of inflammatory diseases, sulphasalazine and the antifolate methotrexate. The identification of SLC19A1 as the major transporter of CDNs into cells has implications for the immunotherapeutic treatment of cancer13, host responsiveness to CDN-producing pathogenic microorganisms11 and—potentially—for some inflammatory diseases.

To systematically identify genes involved in intracellular transport of CDNs, we performed a genome-wide CRISPR interference (CRISPRi) forward genetic screen in the monocytic THP-1 cell line. To visualize STING activation, THP-1 cells were transduced with a CDN-inducible reporter construct (Fig. 1a, b). Consistent with previous results, the synthetic CDN 2′,3′-RR-S2 cyclic di-AMP (2′,3′-RR CDA, Extended Data Fig. 1a) induced a more potent response than 2′,3′-cGAMP13, and the response to both CDNs was severalfold higher than the response to human interferon-β (IFN-β). The response to CDNs was completely dependent on STING expression (Fig. 1b), implying that the reporter primarily reported cell-intrinsic STING activity. For the screen, THP-1 cells expressing a catalytically inactive Cas9 (dCas9) fusion with blue fluorescent protein (BFP) and the transcriptional repression domain KRAB (dCas9–BFP–KRAB), and transduced with a genome-wide guide-RNA (gRNA) library, were stimulated separately with 2′,3′-RR CDA or 2′,3′-cGAMP, and the highest and lowest quartiles of reporter-expressing cells were sorted by flow cytometry before deep-sequencing to identify gRNAs enriched in each population (Fig. 1c, d, Extended Data Figs. 1b, 2, Supplementary Tables 1, 2). The two screens yielded many common hits but there were differences, such as numerous hits in the 2′,3′-cGAMP screen—including STAT2, IRF9, IFNAR1 and IFNAR2 (Supplementary Table 2). Hence, the 2′,3′-RR CDA screen may have mostly been dependent on intrinsic STING signalling, whereas

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Fig. 1 | Genome-wide CRISPRi screen for host factors necessary for stimulation by CDNs. a. Schematic overview of the tdTomato reporter construct. tdTomato expression is driven by interferon-stimulatory response elements (ISRE) followed by a mouse minimal interferon-β (mmIFN-β) promoter. b. Control THP-1 cells and STING-depleted (STING KD) THP-1 cells were incubated with 2′,3′-RR CDA (100 ng ml⁻¹), 2′,3′-cGAMP (10 μg ml⁻¹) or human IFN-β (100 ng ml⁻¹). After 24 h, tdTomato reporter expression was analysed by flow cytometry. Data are representative of three independent experiments with similar results. c. Schematic overview of the genome-wide CRISPRi screen. A genome-wide library of CRISPRi gRNA-expressing THP-1 cells was stimulated with CDNs. Twenty hours after stimulation, cells were sorted into a tdTomatohigh group (lowest 25% of cells) and a tdTomato low group (highest 25% of cells). DNA from the sorted cells was deep-sequenced to reveal gRNA enrichment in the two groups. d, e. Distribution of the robust rank aggregation (RRA) score in the comparison of hits enriched in the tdTomatohigh versus tdTomato low groups of THP-1 cells stimulated with 2′,3′-RR CDA (d) or 2′,3′-cGAMP (e). Each panel represents combined results of two independent screens. AU, arbitrary units.
the 2′3′-cGAMP screen may have been partly dependent on autocrine-paracrine IFN-β signalling.

In both screens, the top hits in the hypo-responsive population (that is, the genes that are most important for robust responses to CDNs) included the gene for the transcription factor IRF3, which acts directly downstream of STING. A gRNA for STING (also known as TMEM173) was also enriched in hypo-responsive cells from both screens, but other STING gRNAs were not enriched—presumably because they were ineffective at interfering with STING expression (Supplementary Tables 1, 2).

**SLC19A1** was one of the most significant hits in both screens. SLC19A1 is a folate–organic phosphate transporter that transports folates, structurally similar antifolates and a variety of organic phosphates encompassing (among others) thiamine derivatives and nucleotides. Folate import is coupled to organic phosphate export and many inhibition and exchange phenomena have previously been demonstrated.

To validate the role of SLC19A1 in CDN stimulation, the top two enriched SLC19A1-targeting gRNAs from the 2′3′-RR CDA screen were used to stably deplete SLC19A1 in THP-1 cells expressing dCas9–KRAB (Extended Data Fig. 3a). SLC19A1-depleted cells grew normally, but uptake of the SLC19A1 substrate methotrexate was nearly abolished in these cells (Extended Data Fig. 3b). Similar to IRF3-depleted cells, SLC19A1-depleted THP-1 or U937 cells (Extended Data Fig. 3b) were defective in reporter responses induced by 2′3′-cGAMP, 2′3′-cGAMP and 3′3′-CDA (a bacterial CDN that stimulates STING), but responded normally to IFN-β stimulation (Fig. 2a, b, Extended Data Fig. 3d–f). SLC19A1 depletion—similar to depletion of IRF3 or STING—also inhibited CDN-induced expression of direct downstream target genes in the STING pathway, including IFNB1, CCL5 and CXCL10 and the chemokine genes with increasing concentrations of the competitive inhibitors methotrexate, 5-me-THF or DMSO as vehicle control, before stimulation with 2′3′-RR CDA (1.25 μg ml⁻¹), 2′3′-cGAMP (15 μg ml⁻¹) or IFN-β (100 ng ml⁻¹).

Cells were analysed as in a. For each stimulant, the data were normalized to the DMSO controls. In b-d, f, g, data are mean ± s.e.m. of n = 3 biological replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett’s test for the comparison to stimulated control cells (b–d), unpaired two-tailed Student’s t-tests (e) or two-way ANOVA followed by uncorrected Fisher’s least significant difference tests (f). P = 0.0002, P = 0.0013, P = 0.0005, P = 0.0006 and ****P ≤ 0.0001. NS, not significant.

**SLC19A1** overexpression robustly increased CDN responsiveness in wild-type THP-1 cells and in cell lines that normally respond poorly.
SLC19A1 is critical for STING-dependent responses to exogenous CDNs but not when CDNs are provided intracellularly.

**a**, Immunoblot analysis of protein expression and phosphorylation in control THP-1 cells or THP-1 cells expressing the indicated CRISPRi gRNAs. Cells were stimulated for 2 h with 10 μg/ml 2′3′-RR CDA (RR CDA) or left unstimulated. p-TBK1, TBK1 phosphorylated on Ser172; p-IRF3, IRF3 phosphorylated on Ser396; p-STING, STING phosphorylated on Ser172; p-IRF3, IRF3 phosphorylated on Ser396; p-STING, STING phosphorylated on Ser366. **b**, Control (CTRL) THP-1 cells or SLC19A1-depleted THP-1 cells were transfected with increasing amounts of interfering-stimulatory DNA (ISD) for 3 h and induction of IFNBI mRNA was measured by quantitative PCR with reverse transcription (RT–qPCR). c, Cells were stimulated as in a in the absence or presence of digitonin (5 μg/ml−1). d, Control THP-1 cells were stimulated as in c, with the addition of the indicated SLC19A1 inhibitors (all at 750 μM): methotrexate (MTX), 5-methy-THF (THF), sulfasalazine (SSZ) or DMSO (as vehicle control). TR, transferrin receptor. Data in **a**, **c**, and **d** are representative of n = 3 biological replicates. For gel source data, see Supplementary Fig. 1. In **b**, data are mean ± s.e.m. of n = 3 biological replicates and statistical analyses were performed using two-way ANOVA followed by post hoc Sidak’s test.

(continued)

**b** shows the same panels as **a** but with the addition of the indicated SLC19A1 inhibitors (all at 750 μM): methotrexate (MTX), 5-methy-THF (THF), sulfasalazine (SSZ) or DMSO (as vehicle control). Data in **b** are mean ± s.e.m. of n = 3 biological replicates and statistical analyses were performed using two-way ANOVA followed by post hoc Sidak’s test.

**c** and **d** show the same panels as **a** and **b**, respectively, but with the addition of the indicated SLC19A1 inhibitors (all at 750 μM): methotrexate (MTX), 5-methy-THF (THF), sulfasalazine (SSZ) or DMSO (as vehicle control). Data in **c** and **d** are mean ± s.e.m. of n = 3 biological replicates and statistical analyses were performed using two-way ANOVA followed by post hoc Sidak’s test.

To directly assess the effect of SLC19A1 on STING pathway activation (Extended Data Fig. 5a), we used immunoblotting to evaluate phosphorylation of STING, IRF3 and TBK1 induced by a 2-h exposure to 2′3′-RR CDA in control (non-targeting gRNA) versus CRISPRi-depleted cells (Fig. 3a). As expected, STING depletion inhibited phosphorylation of both TBK1 and IRF3, whereas IRF3 depletion did not inhibit phosphorylation of TBK1 or STING. By contrast, SLC19A1 depletion resulted in major defects in phosphorylation of STING, TBK1 and IRF3, which indicates that SLC19A1 acts upstream of STING.

Protein levels of STING, TBK1 and IRF3 were unaltered in SLC19A1-depleted cells. Notably, SLC19A1-depleted cells responded normally when transfected with DNA (Fig. 3b). Furthermore, when transport was bypassed by permeabilizing SLC19A1-depleted cells with digitonin, STING exhibited a normal phosphorylation response to CDNs (Fig. 3c). Similarly, the inhibitory effects on CDN-induced STING phosphorylation and gene expression of SLC19A1 blockers—including methotrexate, 5-me-THF and the irreversibly, covalent SLC19A1 inhibitor N-hydroxysuccinimidime (NHS)–methotrexate (Methods)—were bypassed when the cells were permeabilized with digitonin (Fig. 3d, Extended Data Fig. 5b–f). Thus, STING functioned normally in SLC19A1-depleted or inhibited cells when CDNs or DNA were introduced directly into the cytosol, consistent with a role for SLC19A1 in CDN transport.

To directly test the effect of SLC19A1 on transport, we monitored cellular uptake of 32P-labelled CDNs (Extended Data Fig. 6a–h). SLC19A1 overexpression resulted in a two-to-threefold enhancement of [32P]2′3′-cGAMP uptake by THP-1, K562 and C1R cells (Fig. 4a, b, Extended Data Fig. 6i). Conversely, SLC19A1 depletion reduced the uptake of [32P]2′3′-cGAMP in THP-1 and K562 cells (Fig. 4a, b, Extended Data Fig. 6j–m). Furthermore, we observed reduced 2′3′-cGAMP influx in THP-1 and K562 cells treated with NHS–methotrexate (Fig. 4c). Uptake of 2′3′-cGAMP by THP-1 cells was also inhibited by excess unlabelled 2′3′-cGAMP as well as by the bacterial CDNs 3′3′-cGAMP, 3′3′-CDA and 3′3′ c-di-GMP. Thus, CDN interactions with the transporter are not highly specific for the 2′3′ linkage or the specific nucleotides (Fig. 4d).

The nucleoside monophosphates AMP and GMP—the major ENPP1 hydrolysis products of 2′3′-cGAMP—slightly inhibited uptake of [32P]2′3′-cGAMP (Fig. 4d), consistent with previous observations that AMP, other nucleotides and organic phosphates in general inhibit SLC19A1-mediated transport17,18. Our findings indicate that SLC19A1 broadly interacts with CDNs irrespective of the phosphodiester linkages or the base content, but that breakdown products of CDNs have a limited effect on CDN uptake.

Sustained inhibition of 2′3′-cGAMP uptake occurred when folinic acid, 5-methotrexate or sulfasalazine were added to the culture medium of various cell lines (Fig. 4e, Extended Data Fig. 6n–o). In terms of half-maximum inhibitory concentration (IC50), sulfasalazine (IC50 = 2.1 μM) and folinic acid (IC50 = 4.8 μM) were in the same range as 2′3′-cGAMP (IC50 = 1.89 μM) (Extended Data Fig. 6p), similar to the dosage of 2′3′-cGAMP required for STING activation and interferon responses (10–20 μM) (Extended Data Fig. 3d). Consistent with their much higher affinity for SLC19A1 binding compared with folinic acid, 5-meth-THF (IC50 = 4.1 nM) and methotrexate (IC50 = 54.8 nM) were much more potent inhibitors of CDN uptake17 (Extended Data Fig. 6p). Notably, 5-meth-THF incompletely inhibited [32P]2′3′-cGAMP uptake, unlike the other folates or antifolates. Consistent with an antiporter mechanism of uptake, preloading the cells with 5-meth-THF—which trans-stimulates SLC19A1 import12—augmented 2′3′-cGAMP influx (Extended Data Fig. 6q). These observations establish that CDN import is altered by known substrates and inhibitors of the SLC19A1 transporter in a wide range of human cell lines.

We next monitored 2′3′-cGAMP uptake in primary healthy adult human peripheral blood mononuclear cells (PBMCs). Treatment of PBMCs with NHS–methotrexate, or excess, unlabelled 5-meth-THF, methotrexate or sulfasalazine strongly inhibited [32P]-2′3′-cGAMP uptake (Fig. 4f, Extended Data Fig. 6r). These data generalize our findings to normal human blood cells.

In contrast to these results in human cells, neither CDN uptake nor CDN-induced Cxcl10 expression was inhibited by depleting Slc19a1 expression in the mouse C1498 or L1210 cell lines, the latter of which has extensively been studied in the context of SLC19A1-mediated transport17,18,25 (Extended Data Fig. 7). Slc19a1 depletion in mouse bone marrow–derived macrophages or dendritic cells also did not block Ifng gene expression induced by CDNs (Extended Data Fig. 7i–l). Furthermore, antifolates that inhibit SLC19A1, including methotrexate, did not inhibit 2′3′-cGAMP uptake by mouse splenocytes (Extended Data Fig. 7m–o). Collectively, these results suggested that SLC19A1 expression and function are essential for uptake of the metazoan CDN 2′3′-cGAMP by human cell lines and human primary cells ex vivo, but not by the mouse cells we studied. Therefore, mouse cells probably express another potent CDN transporter.

SLC19A1-mediated import of CDNs would require a direct interaction with the CDN. Consistent with this hypothesis, His-tagged
SLC19A1 was precipitated by 2′3′-cGAMP immobilized on Sepharose beads (Fig. 4g, Extended Data Fig. 8a, b). This interaction was competitively disrupted by free, unbound 2′3′-cGAMP and 3′5′-CD4 (Fig. 4h, Extended Data Fig. 8c) or by 5-me-THF and methotrexate (Fig. 4h). These data suggest that CDNs interact with SLC19A1, consistent with its proposed role as a CDN transporter. Taken together, our results demonstrate that SLC19A1 is a CDN transporter in human cells, which is required for activation of type I interferon mediated by exogenous CDNs.

The response to CDNs was relatively weak in most cell lines that we tested and was increased by overexpression of SLC19A1 or permeabilization of cells (Figs. 2f, 3c). Among a large set of cell lines, THP-1 cells are among the highest in expression of both SLC19A1 and STING (Extended Data Fig. 9). It is likely that expression of SLC19A1 and STING are each important predictors of the responsiveness of cell lines and tumours to CDN stimulation.

Methotrexate, folic acid and sulfasalazine almost completely blocked CDN uptake and/or stimulation, whereas CDN stimulation was not completely inhibited in SLC19A1-null cells. This implies that another transporter sensitive to these drugs may have a role in CDN uptake. Although it was not a hit in our screen, overexpression of SLC46A1, which encodes the only other known folate transporter 15, increased responses to CDNs (Extended Data Fig. 10a). However, depletion of SLC46A1 (approximately 90% reduction in mRNA) had little or no effect on CDN stimulation, even when combined with depletion of SLC19A1 (Extended Data Fig. 10c). Overexpression of SLC46A3, another hit in our screen, increased the response to CDNs (Extended Data Fig. 10b) and depletion of SLC46A3 (90% effective) had a minor effect on reporter induction by both CDNs (Extended Data Fig. 10d). However, depleting both SLC19A1 and SLC46A3 together did not reduce responses more than depletion of SLC19A1 alone (Extended Data Fig. 10d), which suggests that neither SLC46A3 nor SLC46A1 is responsible for most of the residual CDN transport in SLC19A1-depleted cells.

Our findings extend the spectrum of organic phosphates that use SLC19A1 to 2′3′-cGAMP, 2′3′-RR CDA and probably other CDNs, by the direct measurement of their transport and the effect of extracellular and intracellular folates on their uptake in human cells by this route. In this context, SLC19A1 could have an important role in the antitumour and adjuvant effects of CDNs administered to patients. It may also be important in cell-to-cell transport of CDNs in immune responses or immune pathology. For example, the SLC19A1 inhibitors methotrexate and sulfasalazine are first-line treatments in rheumatoid arthritis and are widely used to treat inflammatory bowel diseases, including ulcerative colitis and Crohn’s disease 27-29. Although no direct evidence for this is available in humans, studies in mouse models of inflammatory bowel disease raise the possibility that host cells import CDNs produced by intestinal bacteria, activating STING in a cGAS-independent fashion 30. It remains to be determined whether SLC19A1 has a role in such processes in humans. In conclusion, we have identified
SLC19A1—*in humans*—as a CDN transporter with potential relevance in the context of cancer immunotherapy and immunopathology.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgments, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-019-1553-0.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Cell culture. All cell lines were cultured at 37°C in humidified atmosphere containing 5% CO₂ with medium supplemented with 100 µM linoleic acid, 100 µg ml⁻¹ streptomycin, 0.2 mg ml⁻¹ gentamycin, 10 µg ml⁻¹ sodium taurocholate, 200 µM HEPES, and 10% FCS. THP-1, C1R, and K562 cells were cultured in RPMI medium, and 293T, 293T transfected with human STING (293T-hSTING), MDA-MBA-453 (MDA) and RAW 264.7 macrophages were cultured in DMEM medium. Human embryonic kidney 293F cells (HEK293F) grown in FreeStyle 293 medium supplemented with GlutaMax (GIBCO) at 37°C in the presence of 5% CO₂ in a shaking incubator. THP-1, K562, 293T cells, and RAW 264.7 macrophages were from existing stocks in the laboratory. MDA-MB-231 were obtained from the ATCC. C1R cells were a gift from Y. Spies (Fred Hutchinson Cancer Research Center). HEK293F cells were a gift from D. Veesler (University of Washington). The 293T-hSTING cells were generated as previously described. L1210 cells were obtained from ATCC (CCL-219) and cultured in DMEM including 10% horse serum (Gibco, cat. no. 26-050-088). L1210 cells were authenticated by ATCC. MDA-MBA-453 were authenticated by the Berkeley Cell Culture Facility using karyotyping and/or PCR. Other cell lines were not authenticated. All cell lines were negative for mycoplasma contamination.

Antibodies and reagents. The following antibodies were obtained from Cell Signaling Technology: rabbit-anti-human TBK1 monoclonal (clone D1B4; 1:500 for immunoblot), rabbit-anti-human p-TBK1 monoclonal clone D52C2; 1,000 for immunoblot), rabbit-anti-human STING monomonal (clone D2P2F; 1:2,000 for immunoblot), rabbit-anti-human p-STING monoclonal (clone D7C35; 1:1,000 for immunoblot), rabbit-anti-human p-IRF3 monoclonal (clone 4D4; 1:1,000 for immunoblot). Antibodies obtained from LI-COR Biosciences: goat-anti-mouse IgG IRDye 680RD conjugated (cat. no. 926-68073; used at 1:5,000), donkey-anti-rabbit IgG IRDye 800CW conjugated (cat. no. 926-32213; used at 1:5,000), and donkey-anti-rabbit IgG IRDye 680RD (cat. no. 926-68073; used at 1:5,000). Other antibodies: rabbit-anti-human IFN-γ monoclonal (Biolegend, clone JS1; 1:500 for flow cytometry), mouse-anti-human CD55 monoclonal (Thermo Fisher Scientific, clone L5H10; 1:1,000 for immunoblot), rabbit-anti-human CDCA1 polyclonal (BosterBio, cat. no. PB9050; 0.4 µg ml⁻¹ for immunoblot), rabbit-anti-human CD55 monoclonal (Biolegend clone pS2; 1:250 for flow cytometry), mouse-anti-human CD95 monoclonal (Biolegend clone pS2; 1:250 for flow cytometry). Reagents used: 5-µmthyl THF (Cayman Chemical, cat. no. 161159), methotrexate (Cayman Chemical, cat. no. 13966), folinic acid (Cayman Chemical, cat. no. 20515), sulforasazine (Sigma-Aldrich, cat. no. S0883), polyclene (EMD Millipore, cat. no. TR100G), 3′′-cyclic-di-AMP (3′′-c-di-AMP) (InviBio, cat. no. 1250-3), 2′′,3′′-cyclic-di-AMP (2′′,3′′-c-di-AMP) (Thermo Fisher Scientific, cat. no. 20515), 2′′,3′′-cyclic-di-GMP (2′′,3′′-c-di-GMP) (InviBio, cat. no. RG0713) (gifts from Aduro Biotech), human IFN-β1 (PeproTech, cat. no. 300-02B), mouse IFN-γ1 (BioLegend, cat. no. 581302). Antibody selection: puromycin (Sigma-Aldrich, cat. no. P8833), blasticidin (Invivogen, cat. no. ant-bl-1; used at 10 µg ml⁻¹), zeocin (Invivogen, cat. no. ant-zeo-1; used at 200 µg ml⁻¹).

Plasmids. A gBLOCK gene fragment (Integrated DNA Technologies) encoding the tdTomato reporter gene driven by the ISREs and the minimal mouse IFN-γ promoter was cloned into a dual-promoter lentiviral plasmid using Gibson assembly. This lentiviral plasmid co-expressed the zeocin resistance gene and GFP via a T2A ribosomal-skipping sequence controlled by the human EF1α promoter, and was generated as previously described.

For rescue and overexpression of the folate-organic phosphate antipporter SLC19A1, the transfection of target cells with plasmids encoding transmembrane domainless human ORP1L (hORP1L), which is a target protein for CRISPRi gRNAs, was performed. The constructs were transfected using Lipofectamine 2000 (Invitrogen). The cell lines used for this study were MDA-MBA-453 and MCF-7. The cell lines were cultured in DMEM medium supplemented with 10% FCS and 1% antibiotic/antimycotic. The cells were transfected in triplicate and analyzed 48–72 h post-transfection. The GFP expression was quantified by flow cytometry using FACS Canto II (BD Biosciences). Each sample was analyzed in triplicate, and the mean value was used for statistical analysis. The data were analyzed using the GraphPad Prism software (version 5.0). The results were expressed as the fold change in enrichment compared to the control. The statistical significance was determined using the Student's t-test. The data are presented as mean ± SD.

Screen data analysis. CRISPRi samples were analysed using the Python-based ScreenProcessing pipeline (https://github.com/mhlorbeck/ScreenProcessing). Normalization using a set of negative control genes and calculations of phenotypes and Mann–Whitney P values were performed as previously described. In brief, Illumina 50-bp single-end sequencing reads for pooled sublibraries 1 to 4 and 5 to 7 were trimmed to 29 bp and guides were quantified by counting exact matches to the CRISPRi v2 human library guides. Phenotypes were calculated as the log₂ fold change in enrichment of an sgRNA in the high and low samples relative to the control sample. The significance was determined using the Mann–Whitney test. The filters were applied to each sample and then to all samples. The significance was determined using the Bonferroni correction. The screening data were analyzed using the CRISPResso tool (https://crispresso.gforge.inria.fr) to identify significant genes. The identified genes were further analyzed using the clusterProfiler R package (version 3.12.2) to identify enriched pathways and pathways with significant enrichment. The pathways were visualized using the Cytoscape software (version 3.7.0) and the NetworkAnalyst platform (version 4.6). The significance of the enrichment was determined using the hypergeometric test. The results were expressed as the fold change in enrichment compared to the control. The statistical significance was determined using the Student's t-test. The data are presented as mean ± SD.
Table 2). We integrated multiple gRNAs per gene comparing the hypo-responsive and hyper-responsive populations calculated as RNA scores as depicted in Fig. 1d. Similar results were obtained when each sorted population was compared to unsorted cells (Supplementary Table 2).

Genes were also ranked by individual gRNAs with the greatest enrichment or depletion between the hypo-responsive and hyper-responsive libraries. gRNA read counts were normalized to library sequencing depth by converting to read counts per million total reads. For each gRNA, the ratio between the read counts for the hypo-responsive and hyper-responsive libraries was determined and averaged between replicates. For hypo-responsive gene rankings, each gene was ranked by the single corresponding gRNA with the highest hypo-to-hyper ratio (Supplementary Table 1, ‘highest ratio hypo/hyper’ column). For hyper-responsive gene rankings, each gene was ranked by the single corresponding gRNA with the lowest hypo-to-hyper ratio (Supplementary Table 1, ‘lowest ratio hypo/hyper’ column). Gene-level phenotypes are available in Supplementary Tables 1, 2.

**CDN and IFN-β stimulation assays.** The week before stimulation experiments, cells were cultured at the same density. The day before stimulation, cells were seeded to 0.5 × 10^6 cells per ml. Cells were stimulated with CDN or IFN-β 3 in 48-well plates using 50,000 cells per well in 300 μl medium. After 18–24 h, cells were transferred to a 96-well plate and tdtomato expression was measured by flow cytometry using a high-throughput plate reader on a BD LSR Fortessa. For stimulations in the presence of sulfasalazine, 5–me-THF or methotrexate, cells were stimulated in 48-well plates using 20,000 cells per well in 300 μl medium. Cells were incubated with compounds or DMSO as vehicle before stimulations with CDN or IFN-β 3. 18–24 h after stimulation, tdtomato reporter expression was quantified by flow cytometry using a high-throughput plate reader on a BD LSR Fortessa.

**Production of SLC19A1-knockout cell lines.** As an alternative approach to corroborate the role of SLC19A1 in CDN responses, SLC19A1 was disrupted in THP-1 cells using the conventional CRISPR-Cas9 system. THP-1 cells expressing the tdtomato reporter were transduced with a CRISPR-Cas9 lentiviral plasmid encoding a control gRNA or a gRNA targeting SLC19A1 at a region critical for transport 4 (Supplementary Table 3). Transduced cells were selected using puromycin for two days and single-cell-selected using a BD FACSAria cell sorter. Control cells and SLC19A1-targeted cells were selected that had comparable forward and side scatter by flow cytometry analysis. Genomic DNA was isolated from clones using the Qiamp DNA minikit (Qiagen), and the genomic region surrounding the SLC19A1 gRNA target site was amplified by PCR using primers 5′-TTTCTTCCAGCCTCAACTACTCTC-3′ and 5′-CAGATCCGGGCCAGCAGCTGATG-3′. PCR product was cloned into pBluescript host vector to create a ligation-independent cloning (LIC) cassette. Gating strategies for screening and visualizing using a Typhoon scanner.

**shRNA knockdown.** For shRNA knockdown experiments, shRNA sequences were cloned into the pLKO.1 lentiviral expression vector. Cells were transduced with lentivirus and selected using 2 μg ml⁻¹ puromycin for at least 5 days. As controls, shRNAs targeting GFP (shRNA1; TRCN00000231753) and luciferase (shRNA2 TRCN0000231757) were used. Mouse SLC19A1 targeting shRNA sequences: GACGCTGAGCTACTAGATT (shRNA 4), and CGTATCTTATCTATATCTT (shRNA 6); human SLC19A1 targeting shRNA (shRNA 9): CGACGGTGTTCCAGATTGAA. All shRNA knockdowns were confirmed by real-time qPCR. Depletion of SLC19A1 function was confirmed by showing decreased uptake of [3H]-methotrexate (see below and Extended Data Figs. 6l, 7c, g). shRNA knockdown experiments were performed using the iScript cDNA synthesis kit (Bio-Rad). TaqMan real-time qPCR assays were used for quantification of human IFNB1 (Hs01077958_s1), human OASL (Hs00984387_m1), and human ISG15 (Hs01921425_s1). Actb (Hs01060666_g1) served as an endogenous control.

**Nucleotide-binding assays.** The ability of radiolabelled 2′,3′-cGAMP and 3′,3′-cGDA to bind recombinant STING was evaluated by differential radial capillary action of ligand assay (DraCALA) analysis, as previously described 4 (Extended Data Fig. 6d). In brief, varying concentrations of recombinant STING were incubated with about 1 nM of radiolabelled CDN in binding buffer for 10 min at room temperature. The reaction mixtures were spotted on nitrocellulose membranes and air-dried for 15 min. The membranes were then exposed to a PhosphorImager screen and visualized using a Typhoon scanner.

**NHS–methotrexate synthesis and affinity labelling.** NHS–methotrexate was prepared as previously described 41. In brief, methotrexate (2.2 mg) was acidified by the addition of HCI and dried under vacuum. Next, the sample was incubated with HisPur Ni-NTA resin (Thermo Scientific) for 30 min to remove recombinant CDN. The resulting slurry was transferred to a minispin column (Thermo Scientific) to elute crude [32P]2′,3′-cGAMP. Recombinant mSTING-CDT protein was used for further purification of synthesized [32P]2′,3′-cGAMP. mSTING-CDT (100 μM) was bound to HisPur Ni-NTA resin and incubated with the remaining crude 2′,3′-cGAMP synthesis reaction mixture for 30 min on ice. Following removal of the supernatant, the Ni-NTA resin was washed three times with cold binding buffer. The resin was then incubated with 100 μl of binding buffer for 10 min at 95 °C, and transferred to a minispin column to elute [32P]2′,3′-cGAMP. The resulting STING-purified [32P]2′,3′-cGAMP was purified by TLC analysis and determined to be about 98% pure (Extended Data Fig. 6c).

**RadioLabeled 3′,3′-cGDA was synthesized as previously described 41. In brief, 1 μM 3′,3′-cGAMP was incubated with 1 μM of recombinant Disa in binding buffer at 37° C overnight. The reaction mixture was boiled for 5 min at 95°C and Disa was removed by centrifugation. Recombinant His-tagged RECON was then used to further purify the 3′,3′-cGAMP reaction mixture. One-hundred micromolar His-tagged RECON was bound to HisPur Ni-NTA resin for 30 min on ice. The resin was washed three times with cold binding buffer and then incubated with 100 μl of binding buffer for 5 min at 95°C. The slurry was then transferred to a minispin column to elute [32P]2′,3′-cGAMP. The purity of the radiolabeled 3′,3′-cGAMP was assessed by TLC and determined to be about 98%.

**Data availability.** All data generated or analyzed during this study are included in this published article and its Supplementary Information.
2′,3′-cGAMP was incorporated into THP-1 cells at a linear rate over at least three hours of incubation (Extended Data Fig. 6e), during which time the [32P]2′,3′-cGAMP was not hydrolyzed or modified (Extended Data Fig. 6f). 2′,3′-cGAMP uptake was most efficient at a pH range of 7.5 to 8.0 in both THP-1 and U937 cells (Extended Data Fig. 6g, h), consistent with a neutral pH optimum for SLC19A1.15

**-mRNA extraction**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using the LightCycler 480 II (Roche) with the following primers: SLC19A1 (Forward: 5′-ATG TGC CTC CAC GAC AGA C-3′, Reverse: 5′-GAG GGT GCA AGG GAA TTA AG-3′), GAPDH (Forward: 5′-GGA GGT GGA GGT GAT GGA G-3′, Reverse: 5′-TCA GCT GAT GGT GAT GCA G-3′).

**Western Blot Analysis**

Whole cell lysates were prepared using lysis buffer (50 mM HEPES, 300 mM NaCl, 20 mM β-glycerophosphate, 5 mM EDTA, 1% NP-40, 0.1% SDS) containing Complete Regular protease inhibitors. After lysis, samples were mixed with 4X NuPage sample buffer (Invitrogen, cat. no. NP0007), pulsed sonicated and incubated at 75°C. Samples were loaded on a NuPAGE bis-tris polyacrylamide gel (12.5%) and then transferred to a PVDF membrane (Bio-Rad). Membranes were blocked for 1 h with 5% non-fat milk (NFM) and 0.1% Tween 20. Membranes were incubated with primary antibodies overnight at 4°C. After incubation, membranes were washed 3 times with TBS-T wash buffer (20 mM HEPES, 150 mM NaCl, 0.1% Tween 20, 0.1% BSA) and incubated with secondary antibodies at room temperature. Membranes were washed with 2X TBS-T and imaged using Odyssey CLX System (LI-COR). Bands were quantified using ImageJ software.

**Quantitative Real-Time PCR**

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using the LightCycler 480 II (Roche) with the following primers: SLC19A1 (Forward: 5′-ATG TGC CTC CAC GAC AGA C-3′, Reverse: 5′-GAG GGT GCA AGG GAA TTA AG-3′), GAPDH (Forward: 5′-GGA GGT GGA GGT GAT GGA G-3′, Reverse: 5′-TCA GCT GAT GGT GAT GCA G-3′).

**Statistical Analysis**

Results are presented as mean ± standard error of the mean (SEM) and were analyzed using one-way ANOVA with Tukey's post-hoc test. All statistical analyses were performed using GraphPad Prism 7.0 software. A p-value of <0.05 was considered statistically significant.
Dickinson) from healthy adults with written informed consent. Bulk PBMCs were isolated using SepMate tubes (STEMCELL Technologies) according to the manufacturer’s instructions. The remaining cells were washed in PBS, resuspended in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) heat-inactivated FBS (HyClone), 10 mM HEPEs, 1 mM sodium pyruvate, 2 mM L-glutamine (Thermo Fisher), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and used immediately for [³²P]adenosine 5’-cGAMP uptake assays. The isolation of primary human cells complied with all relevant ethical regulations and was conducted under a protocol from K. B. Elkon which was approved by the University of Washington Institutional Review Board.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Code availability**
The CRISPRi screen sequences were analysed using the Python-based ScreenProcessing pipeline. This custom code is available at https://github.com/mhorlbeck/ScreenProcessing.

**Data availability**
Raw sequence data from the CRISPRi screens are available at NCBI Gene Expression Omnibus under accession number GSE134371.

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**Author contributions**
R.D.L., S.A.Z. and N.E.G. performed and analysed the experiments; L.O., S.M.M. and G.E.K. assisted with the experiments; S.K.W. and B.G.G. analysed the deep-sequencing data and advised on the screen design; R.D.L., S.A.Z., B.G.G., J.J.W. and D.H.R. designed the experiments and R.D.L., S.A.Z., J.J.W. and D.H.R. prepared the manuscript. All authors critically read the manuscript.

**Competing interests**
D.H.R. is a co-founder of Dragonfly Therapeutics and served or serves on the scientific advisory boards of Dragonfly, Aduro Biotech, and Igne ImmunoTherapy; he has a financial interest in all four companies and could benefit from commercialization of the results of this research. S.M.M. is, and G.E.K. was, an employee of Aduro Biotech.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1553-0.
Correspondence and requests for materials should be addressed to D.H.R.
Reprints and permissions information is available at http://www.nature.com/reprints.
Extended Data Fig. 1 | Structures of CDNs used in this study and gating strategy for the genome-wide CRISPRi screens. a, Structures of the CDNs used in this study. b, Representative gating strategy for flow-cytometry-based sorting of the CRISPRi library of reporter-expressing THP-1 cells stimulated with CDNs. Cells were gated on the basis of their forward scatter (FSC) and side scatter (SSC) using gate P1. The P1 cells were subsequently selected for co-expression of BFP (fluorescent marker for the CRISPRi gRNAs) and GFP (marker for the expression of the reporter construct) (gate P2). Gate P3 excluded cell doublets present among P2 cells. Gate P4 selected for the lowest 25% of cells with respect to tdTomato expression and gate P5 selected for the highest 25%. c, Representative flow cytometry dot plots showing tdTomato expression in unstimulated cells or in cells stimulated for 20 h with cells for 20 h with CDN (2′,3′-RR CDA). Data are representative of \( n = 3 \) biological replicates.
Extended Data Fig. 2 | Results of genome-wide CRISPRi screens for host factors crucial for CDN stimulation. a, b, Volcano plots of the gRNA-targeted genes enriched or depleted in the tdTomato reporter-low versus reporter-high groups after stimulation with 2′,3′-RR CDA (a), or 2′,3′-cGAMP (b). Each panel represents the combined results of two independent screens. Calculations of phenotypes and Mann–Whitney P values were performed as described in Methods.
Extended Data Fig. 3 | SLC19A1 is critical for CDN-induced gene expression. a, b, mRNA expression levels of SLC19A1 (a) or IRF3 (b) in THP-1 cells expressing a CRISPRi vector and a control non-targeting gRNA or gRNAs targeting IRF3 or SLC19A1 (two gRNAs each). c, THP-1 cells described in a and b were exposed to [3H]-methotrexate. After 1 h, radioactivity (in counts per minute, cpm) in lysates of cell pellets was measured. Counts were normalized to protein concentrations in the lysate. d, THP-1 cells expressing a control gRNA or SLC19A1-targeting gRNA were exposed to indicated concentrations of 2′3′-RR CDA or 2′3′-cGAMP. After 20 h, the mean fluorescence intensity (MFI) of tdTomato was quantified by flow cytometry. e, THP-1 cells expressing the indicated CRISPRi gRNAs or non-targeting gRNA (control), were stimulated with IFN-β (100 ng ml⁻¹). After 18–22 h, tdTomato expression was quantified as in Fig. 2a. f, THP-1 cells were incubated with increasing concentrations of the non-competitive inhibitor sulfasalazine or DMSO as vehicle control, before stimulation with 2′3′-RR CDA (1.25 μg ml⁻¹), 2′3′-cGAMP (15 μg ml⁻¹) or IFN-β (100 ng ml⁻¹). After 18–22 h, tdTomato expression was quantified as in Fig. 2a. The data were normalized to the DMSO controls. In a–c and e–f, mean of n = 2 biological replicates are shown. In d, g–k, mean ± s.e.m. of n = 3 biological replicates are shown. Statistical analyses were performed to compare each cell line to the control using a one-way ANOVA followed by post hoc Dunnett’s test (d, g–i) or two-way ANOVA followed by uncorrected Fisher’s least significant difference tests (j). ****P ≤ 0.0001; n.s., not significant.
Extended Data Fig. 4 | SLC19A1 overexpression increases the response to CDNs in various cell lines. Various cell lines expressing a control vector or an SLC19A1 expression vector (SLC19A1 OE) stimulated with 2′3′-cGAMP (10 μg ml⁻¹) (e) or IFN-β (100 ng ml⁻¹) (or 100 ng ml⁻¹ mouse IFN-β in the case of RAW cells). After 20 h, reporter expression was quantified by flow cytometry. Representative flow plots of n = 3 biological replicates shown in Fig. 2f and Extended Data Fig. 3j.)
Extended Data Fig. 5 | Covalent inhibition of SLC19A1 by NHS-methotrexate blocks STING activation. 

a, Schematic overview of CDN-induced phosphorylation (P) of STING and downstream effectors TBK1 and IRF3.

b, THP-1 monocytes pre-treated with DMSO or NHS–methotrexate (5 μM) were treated with varying concentrations of 2′,3′-cGAMP for 4 h, and the amounts of IFNB1 or ISG15 transcripts were measured by RT–qPCR.

c, Semi-native PAGE and immunoblot analysis of STING dimerization and phosphorylation in DMSO and NHS–methotrexate (5 μM) pre-treated THP-1 monocytes stimulated with 100 μM 2′,3′-cGAMP for 4 h. For gel source data, see Supplementary Fig. 1.

d, DMSO and NHS–methotrexate (5 μM)-treated THP-1 monocytes were treated with 100 μM 2′,3′-cGAMP in the presence and absence of digitonin (5 μg ml\(^{-1}\)) for 4 h and the induction of IFNB1 mRNA was measured by RT–qPCR. e, f, DMSO and NHS–methotrexate (5 μM) pre-treated THP-1 monocytes (e) or K562 cells (f) were stimulated for 4 h with 100 μM 2′,3′-cGAMP, or not, in the presence or absence of digitonin (5 μg ml\(^{-1}\)), and the induction of OASL and ISG15 mRNA was measured by RT–qPCR. In b, d–f, data are mean of n = 2 technical replicates and are representative of 3 independent experiments with similar results. In c, data are representative of three independent experiments with similar results.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | SLC19A1 is critical for CDN uptake in human cell lines and primary cells. a, TLC analysis of [32P]ATP standard (STD) and enzymatically synthesized [32P]2’3’-cGAMP (2’3’-cGAMP was purified on STING resin). Unbound nucleotides flowed through the resin (STING FT). Following four washes, the bound [32P]2’3’-cGAMP was eluted over three fractions. b, DRaCALA binding analysis of [32P]2’3’-cGAMP to STING C-CTD in the presence of competing unlabelled nucleotides (200 μM). c, TLC analysis of [32P]ATP and enzymatically synthesized [32P]2’3’-cGAMP or [32P]c-di-AMP to mSTING CTD, determined with DRaCALA assays. Red lines represent the 95% confidence interval for the nonlinear regression. d, Time course of [32P]2’3’-cGAMP uptake by CIR cells transduced with empty vector or SLC19A1 expression vector. e, mRNA expression levels of SLC19A1 (SLC19A1) in K562 cells expressing control shRNAs (sh1 and sh2) or an SLC19A1-targeting shRNA (sh9). f, mRNA expression levels of CXCL10 in K562 cells described in j, stimulated with 5 μg ml⁻¹ 2’3’-RR CDA (RR CDA) for 5 h. g, [3H]-methotrexate uptake in K562 cells described in j, 1 h after exposure to [3H]-methotrexate. h, Time course of [32P]2’3’-cGAMP uptake in K562 cells described in j. i, Time course of [32P]2’3’-cGAMP uptake in U937 monocytes in the presence or absence of 500 μM competing, unlabelled (anti-)folates and sulfasalazine. j, Time course of [32P]2’3’-cGAMP uptake in K562 cells in the presence or absence of 500 μM competing, unlabelled (anti-)folates or sulfasalazine. k, Competition uptake assay of [32P]2’3’-cGAMP uptake in THP-1 cells in the presence of varying concentrations of competing, unlabelled 5-me-THF (IC₅₀ = 4.10 ± 0.16 nM), methotrexate (IC₅₀ = 54.83 ± 5.08 nM), 2’3’-cGAMP (IC₅₀ = 1.89 ± 0.11 μM), sulfasalazine (IC₅₀ = 2.06 ± 0.17 μM), and folic acid (IC₅₀ = 4.79 ± 0.08 μM). q, Trans-stimulation of [32P]2’3’-cGAMP influx in THP-1 cells by 5-me-THF. Cells were preloaded with indicated concentrations of 5-me-THF for 30 min. Cells were washed and incubated with [32P]2’3’-cGAMP for one hour. r, Normalized [32P]2’3’-cGAMP uptake after 1 h in DMSO or NHS–methotrexate (5 μM)-treated human PBMCs from four healthy donors. In a, data are representative of three independent experiments with similar results. In b, data are mean of n = 2 technical replicates and are representative of 3 independent experiments. In c, data are mean of n = 2 technical replicates and are representative of 2 independent experiments. In d, data are mean ± s.d. of n = 3 technical replicates and are representative of 3 independent experiments. In e, data are mean ± s.d. of n = 3 technical replicates and are representative of 3 independent experiments. In g-k, data are mean ± s.e.m. of n = 3 biologically independent experiments. In l, data are mean of n = 2 biologically independent experiments.
Extended Data Fig. 7 | SLC19A1 expression or inhibition has no effect on CDN uptake and signalling in mouse cells. a, mRNA expression levels of Slc19a1 in mouse L1210 cells expressing control shRNAs (sh1 and sh2) or Slc19a1-targeting shRNA (sh4 and sh6). b, mRNA expression levels of Cxcl10 in L1210 cells described in a stimulated with 5 μg ml⁻¹ 2′3′-RR CDA (RR CDA) for 5 h. c, [³H]-methotrexate uptake in L1210 cells described in a 1 h after exposure to [³H]-methotrexate. d, Time course of [³²P]2′3′-cGAMP uptake in L1210 cells described in a. e, mRNA expression levels of Slc19a1 in mouse C1498 cells expressing control shRNAs (sh1 and sh2) or Slc19a1-targeting shRNA (sh6). f, mRNA expression levels of Cxcl10 in the C1498 cells described in e, stimulated with 5 μg ml⁻¹ 2′3′-RR CDA (CDN) for 5 h. g, [³H]-methotrexate uptake in C1498 cells described in e 1 h after exposure to [³H]-methotrexate. h, Time course of [³²P]2′3′-cGAMP uptake in C1498 cells transduced with a non-targeting control shRNA or Slc19a1 shRNA. i, j, mRNA expression levels of Slc19a1 in mouse BMMs (i) or BMDCs (j) not transduced or transduced with control shRNAs (sh1 and 2) or an shRNA targeting Slc19a1. k, l, mRNA expression of the Cxcl10 in cells described in i, j stimulated with 5 μg ml⁻¹ 2′3′-RR CDA (CDN) for 5 h. m, Time course of [³²P]2′3′-cGAMP uptake in primary mouse splenocytes in the presence and absence of 500 μM competing, unlabelled (anti-) folates and sulfasalazine. n, Time course of [³²P]2′3′-cGAMP uptake in primary mouse splenocytes pretreated or not with NHS–methotrexate (5 μM). o, Time course of [³²P]2′3′-cGAMP uptake in L1210 cells pretreated or not with NHS–methotrexate (5 μM). In a–c, e–g, j, l, data are mean of n = 2 biologically independent experiments. In d, h, i, k, n, o, data are mean ± s.d. of n = 3 technical replicates and are representative of 2 independent experiments. In m, data are mean of n = 2 technical replicates and are representative of 2 independent experiments. In time-course experiments (d, h, m–o), data are presented as counts per minute normalized to cell count.
Extended Data Fig. 8 | 2′3′-cGAMP binds to SLC19A1. **a,** Left, SDS–PAGE analysis followed by Coomassie blue staining of His-tagged human SLC19A1 (purified on Ni-NTA) pull-downs with Sepharose beads coupled with 2′3′-cGAMP (+) or control Sepharose beads (−). Input is shown on the right. **b,** Western blots of the samples in a with SLC19A1 antibody. **c,** Pull-downs of SLC19A1 competed with CDNs. His-tagged SLC19A1 was incubated with no CDN or with the indicated competing CDNs (250 μM) before pull-downs with 2′3′-cGAMP-Sepharose, followed by SDS–PAGE and western blotting with SLC19A1 antibody. A pulldown with control Sepharose is shown for comparison. For gel source data, see Supplementary Fig. 1. **d,** SDS–PAGE analysis followed by Coomassie blue staining of pull-downs of mSTING CTD with 2′3′-cGAMP (+) or control (−) Sepharose. In all panels, data are representative of two independent experiments with similar results.
Extended Data Fig. 9 | RNA sequencing data of STING and SLC19A1 mRNA expression in 934 human cancer cell lines available at the Cancer Cell Line Encyclopedia website. Expression is presented as transcripts per kilobase million (TPM). Data are downloaded from the European Bioinformatics Institute Gene expression Atlas (https://www.ebi.ac.uk/gxa/home). The dataset included three of the cell lines we examined, as shown.
Extended Data Fig. 10 | The effect of SLC46A1 and SLC46A3 expression on CDN-induced reporter activation. a, b, Enforced expression of SLC46A1 and SLC46A3 affects the responses of THP-1 cells to CDNs. Control THP-1 cells (transduced with empty expression vector) or SLC46A1-transduced THP-1 cells (a), or control THP-1 cells or SLC46A3-transduced cells (b) were stimulated with 2′3’-RR CDA (1.25 μg ml⁻¹), 2′3’-cGAMP (15 μg ml⁻¹) or IFN-β (100 ng ml⁻¹). tdTomato reporter expression was measured by flow cytometry 18–22 h after stimulation.
c, d, SLC46A1 or SLC46A3 depletions had little or no effects on cellular responses to CDNs, and combining depletion of SLC46A1 or SLC46A3 with SLC19A1 depletion had no additional effect compared to SLC19A1 depletion alone. THP-1 cells were transduced with non-targeting control CRISPRi gRNAs or SLC19A1-targeting CRISPRi gRNA in combination with a second control CRISPRi gRNA or SLC46A1-targeting CRISPRi gRNA in (c) or SLC46A3-targeting gRNA in (d). Cells were stimulated with 2′3’-RR CDA (1.67 μg ml⁻¹), 2′3’-cGAMP (10 μg ml⁻¹) or IFN-β (100 ng ml⁻¹). tdTomato reporter expression was measured by flow cytometry 18–22 h after stimulation. Combined data of three independent experiments. Statistical analysis was performed using unpaired two-tailed Student’s t-tests (a, b) or one-way ANOVA followed by post hoc Tukey’s test when comparing only the effects of depleting SLC46A1 (c) or SLC46A3 (d). Data are mean ± s.e.m. of n = 3 independent replicates.
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Software and code

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Data collection

Sequence data was collected as part of the primary run processing using the innate HiSeq2500 and 4000 control software. Multiplexed sequencing data were demultiplexed using the BCL2Fastq program (version 2.17 and later). Flow cytometry: BD LSR Fortessa, BD LSR Fortessa X20 and BD LSR II. Flow data collection: BD FACSDiva version 6.1.3. RT-qPCR: Bio-Rad C1000 Thermal Cycler; beta counter: LS6500 Liquid Scintillation Counter (Beckman Coulter); Western blot infrared imager: Odyssey CLx System (LI-COR)

Data analysis

the Python-based ScreenProcessing pipeline (https://github.com/mhorlbeck/ScreenProcessing) and MaGeCK version 0.5.2 were used to analyze the CRISPRi sequencing results. For all other statistical analyses, Prism Graphpad 8 was used. Flow data analyses: FlowJo version 10.5.3; RT-qPCR: Bio-Rad CFX Manager 3.1

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Raw sequencing data from the CRISPRi screens are available at NCBI GEO (GEO accession number GSE134371)

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Preliminary experiments were the basis of power calculations, which were based on the magnitude of the preliminary effects and the desired level of statistical significance. The experiments were always repeated, usually multiple times, to ensure reproducibility.

Data exclusions

no data were excluded from the analysis

Replication

the experiments were independently repeated multiple times, and statistical tests have been performed to ensure reproducibility. Information on statistical tests and reproducibility are described in the figure legends.

Randomization

these studies did not involve the comparison of different animals or patients, therefore randomization was not necessary

Blinding

Blinding was not relevant as the reported data was not based on subjective observations, but quantitative measurements, including flow cytometry, radio-activity, fluorescence etc. Nonetheless, samples were allocated an arbitrary number during analyses.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | Unique biological materials |
|     | Antibodies |
|     | Eukaryotic cell lines |
|     | Palaeontology |
|     | Animals and other organisms |
|     | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ChIP-seq |
|     | Flow cytometry |
|     | MRI-based neuroimaging |

Antibodies

Antibodies used

antibody name; supplier name; clone name; catalogue number; lot number; dilution; vendor website

rabbit-anti-human TBK1 mAb; Cell Signaling Technologies; D184; #3504; 4; 1:500; https://www.cellsignal.com/products/primary-antibodies/tbk1-nak-d184-rabbit-mab/3504

rabbit-anti-human phospho-TBK1 mAb; Cell Signaling Technologies; D52C2; #5483; 8; 1:1000; https://www.cellsignal.com/products/primary-antibodies/phospho-tbk1-nak-ser172-d52c2-xp-rabbit-mab/5483

rabbit-anti-human STING mAb; Cell Signaling Technologies; D2P2F; #13647; 1; 1:2000; https://www.cellsignal.com/products/primary-antibodies/sting-d2p2f-rabbit-mab/13647
rabbit-anti-human phospho STING mAb; Cell Signaling Technologies; D7C3S; #19781; 1:1000; https://www.cellsignal.com/products/primary-antibodies/phospho-sting-ser366-d7c3s-rabbit-mab/19781

rabbit-anti-human phospho-IRF3 mAb; Cell Signaling Technologies; 4D4G; #4947; 13; 1:1000; https://www.cellsignal.com/products/primary-antibodies/phospho-irf3-3-ser396-4d4g-rabbit-mab/4947

mouse-anti-β-Actin mAb; Cell Signaling Technologies; 8H10D10; #3700; 15; 1:1000; https://www.cellsignal.com/products/primary-antibodies/b-actin-8h10d10-mouse-mab/3700

goat-anti-mouse IgG IRDye 680RD conjugated; LI-COR Biosciences; polyclonal; #926-68070; c80619-05; 1:5000; https://www.licor.com/bio/reagents/irdye-680rd-goat-anti-mouse-igg-secondary-antibody

donkey-anti-rabbit IgG IRDye 800CW conjugated; LI-COR Biosciences; polyclonal; #926-32213; c80125-15; 1:5000; https://www.licor.com/bio/reagents/irdye-800cw-donkey-anti-rabbit-igg-secondary-antibody

donkey-anti-rabbit IgG IRDye 680RD conjugated; LI-COR Biosciences; polyclonal; #926-68073; c80116-07; 1:5000; https://www.licor.com/bio/reagents/irdye-680rd-donkey-anti-rabbit-igg-secondary-antibody

rabbit-anti-human IRF3 mAb; Abcam; ab76409; #EP2419Y; GR96792-10; 1:2000; https://www.abcam.com/irf3-antibody-ep2419y-ab76409.html

mouse-anti-human transferrin receptor mAb; Thermo Fischer Scientific; H68.4; #13-6800; RB232679; 1:1000; https://www.thermofisher.com/antibody/product/Transferrin-Receptor-Antibody-clone-H68-4-Monoclonal/371-6800

rabbit-ant-human SLC19A1 pAb; BosterBio; polyclonal; #PB9504; 0951512Da210465; 0.4 g/ml; https://www.bosterbio.com/anti-slc19a1-picolband-trade-antibody-pb9504-boster.html

APC-conjugated mouse-anti-human CD55 mAb; BioLegend; JS11; #311311; B183365; 1:50; https://www.biolegend.com/en-us/products/apc-anti-human-cd55-antibody-1792

mouse-anti-human CD59 mAb; BioLegend; p282; #304702; B204936; 1:250; https://www.biolegend.com/en-us/products/purified-anti-human-cd59-antibody-890

APC-conjugated goat-anti-mouse IgG; BioLegend; Poly4053; #405308; B158101; 1:100; https://www.biolegend.com/en-us/products/apc-goat-anti-mouse-igg-minimal-x-reactivity-1383

Validation
All antibodies used in flow cytometry and western blot were bought from commercial vendors and were validated by the manufacturers or relevant literature was cited on their websites (see vendor websites listed above)

Eukaryotic cell lines
Policy information about cell lines

Cell line source(s) THP-1, K562, 293T cells, and RAW macrophages were present in the lab at the time this study began. MDA-MBA-453 cells were obtained from the Berkeley Cell Culture Facility. C1R cells were a generous gift from Veronika Spies (Fred Hutchinson Cancer Center, Seattle WA). 293T+hSTING cells were generated at Aduro Biotech Inc. L1210 cells were obtained from ATCC. 293F cells were a generous gift from David Veesler (University of Washington, Seattle WA)

Authentication L1210 cells were authenticated by ATCC. MDA-MBA-453 were authenticated by the Berkeley Cell Culture Facility using karyotyping and/or PCR, other cell lines were not authenticated

Mycoplasma contamination all cell lines were negative for mycoplasma contamination

Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used

Animals and other organisms
Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals Male and female C57BL/6J mice were obtained from The Jackson Laboratory (aged 4 to 20 weeks)

Wild animals No wild animals were used

Field-collected samples No field-collected samples were used

Human research participants
Policy information about studies involving human research participants

Population characteristics We obtained peripheral blood lymphocytes from unidentified and randomly chosen healthy patients.
Recruitment

The patients were donors.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells were harvested, washed and measured / sorted in RPMI or DMEM media

Instrument

Multicolor flow cytometry was performed on one of the following machines: LSR II, or LSR Fortessa or LSR X20 (BD)

Software

Data was analyzed using FlowJo 10.0

Cell population abundance

Purity was greater than 95% after post sort analysis by flow cytometry

Gating strategy

Representative gating strategy for flow cytometry based sorting of the CRISPRi library of reporter-expressing THP-1 cells stimulated with CDNs. Cells were gated based on their forward scatter (FSC) and side scatter (SSC) using gate P1. P1-population was selected based on the expression of blue fluorescent protein (BFP, fluorescent marker for the CRISPRi gRNAs) and GFP (marker for the expression of the reporter construct) using gate P2. In gate P3, the doublet cells present in gate P2 were excluded. In gate P4, population P3 was gated based on tdTomato expression (see supplemental figure S2)

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.