Expanded View Figures

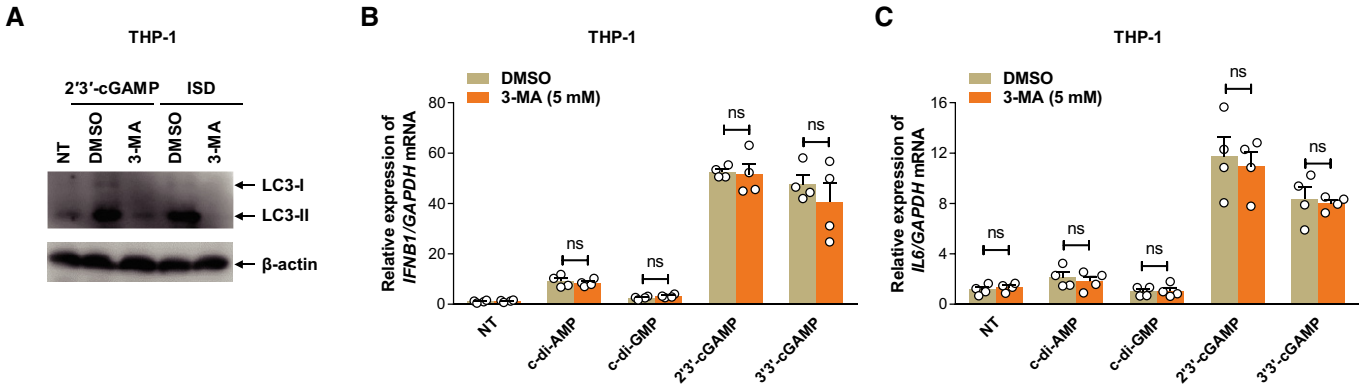

Figure EV1. eCDN-induced type I IFN responses are uncoupled from autophagy.

A Western blot detection of indicated proteins in lysates of THP-1 cells left untreated or treated with ecGAMP or transfected with ISD in the presence of mock or indicated concentrations of 3-MA (5 mM). Data are representative of three independent experiments.

B, C qRT–PCR detection of *IFNB1* (B) and *IL6* (C) mRNA in THP-1 cells stimulated with indicated eCDNs (5 μg/ml) for 4 h in the presence of mock or 3-MA (5 mM). Data are means + SD averaged from four independent experiments performed in technical duplicates, and each symbol represents mean of technical duplicates. Two-way ANOVA followed by Bonferroni's *post hoc* test was used for statistical analysis. ns, not significant.

Source data are available online for this figure.

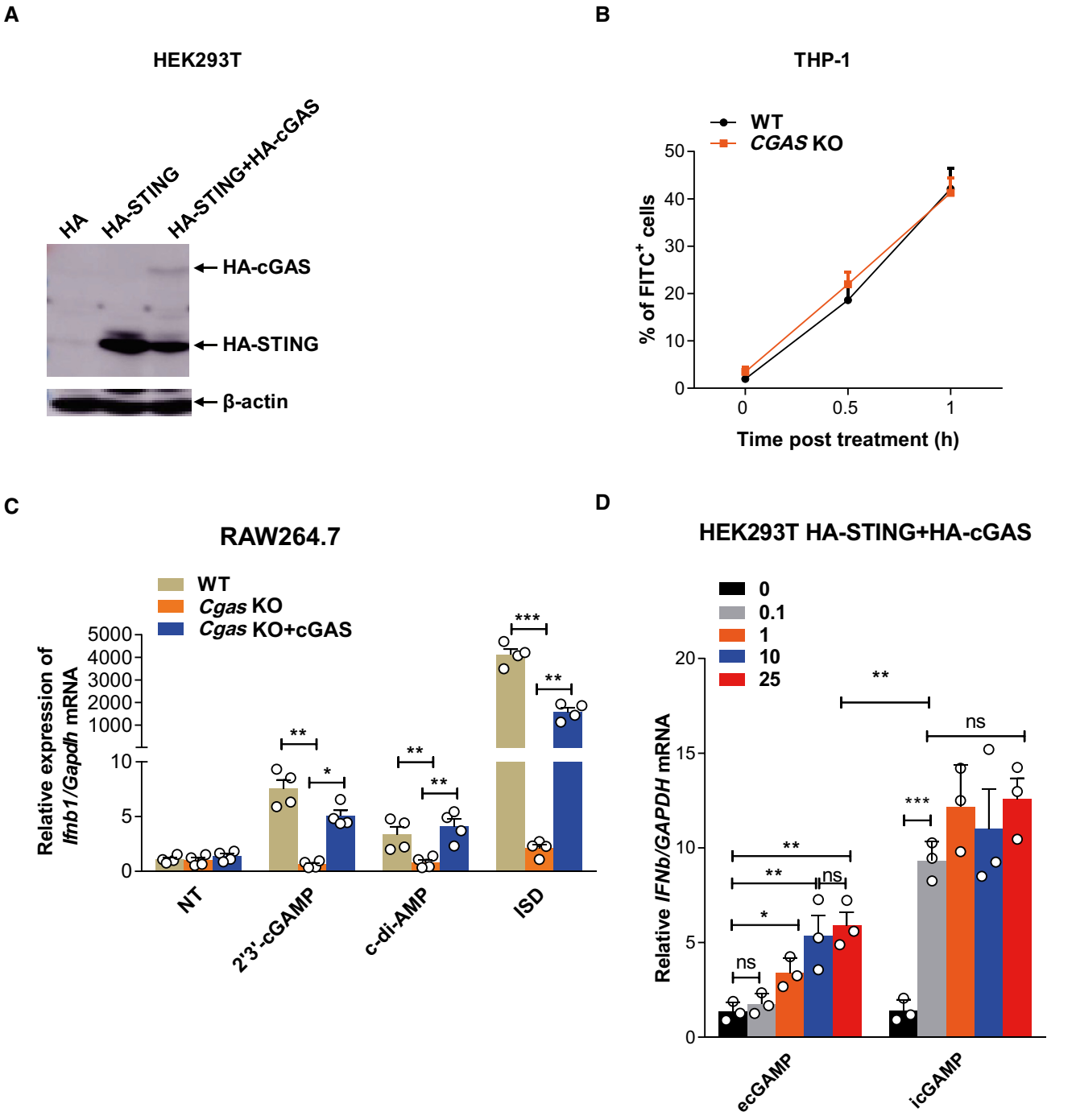

Figure EV2. cGAS contributes to sensing of eCDNs.

A Western blot detection of indicated proteins in lysates of HEK293T cells stably transfected with pcDNA3.1-HA (HA), HA-STING, and HA-STING + HA-cGAS. Data are representative of three independent experiments.

B Frequencies of FITC$^+$ WT and *CGAS* KO THP-1 cells stimulated with FITC-ecGAMP (5 μg/ml) for indicated times.

C qRT–PCR detection of *Ifnb1* mRNA in WT, *Cgas* KO, or *Cgas* KO complemented with cGAS (*Cgas* KO+cGAS) RAW264.7 cells stimulated with indicated eCDNs (5 μg/ml) or transfected with ISD.

D qRT–PCR detection of *IFNB1* mRNA in HEK293T cells stably transfected with both HA-STING and HA-cGAS stimulated with either ecGAMP or icGAMP at the indicated concentrations (μg/ml) for 24 h.

Data information: Data are means + SD averaged from at least three independent experiments performed with technical triplicates. Each symbol in panels (C, D) represents the mean of technical triplicates. Two-way ANOVA followed by Bonferroni's *post hoc* test was used for statistical analysis. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Source data are available online for this figure.

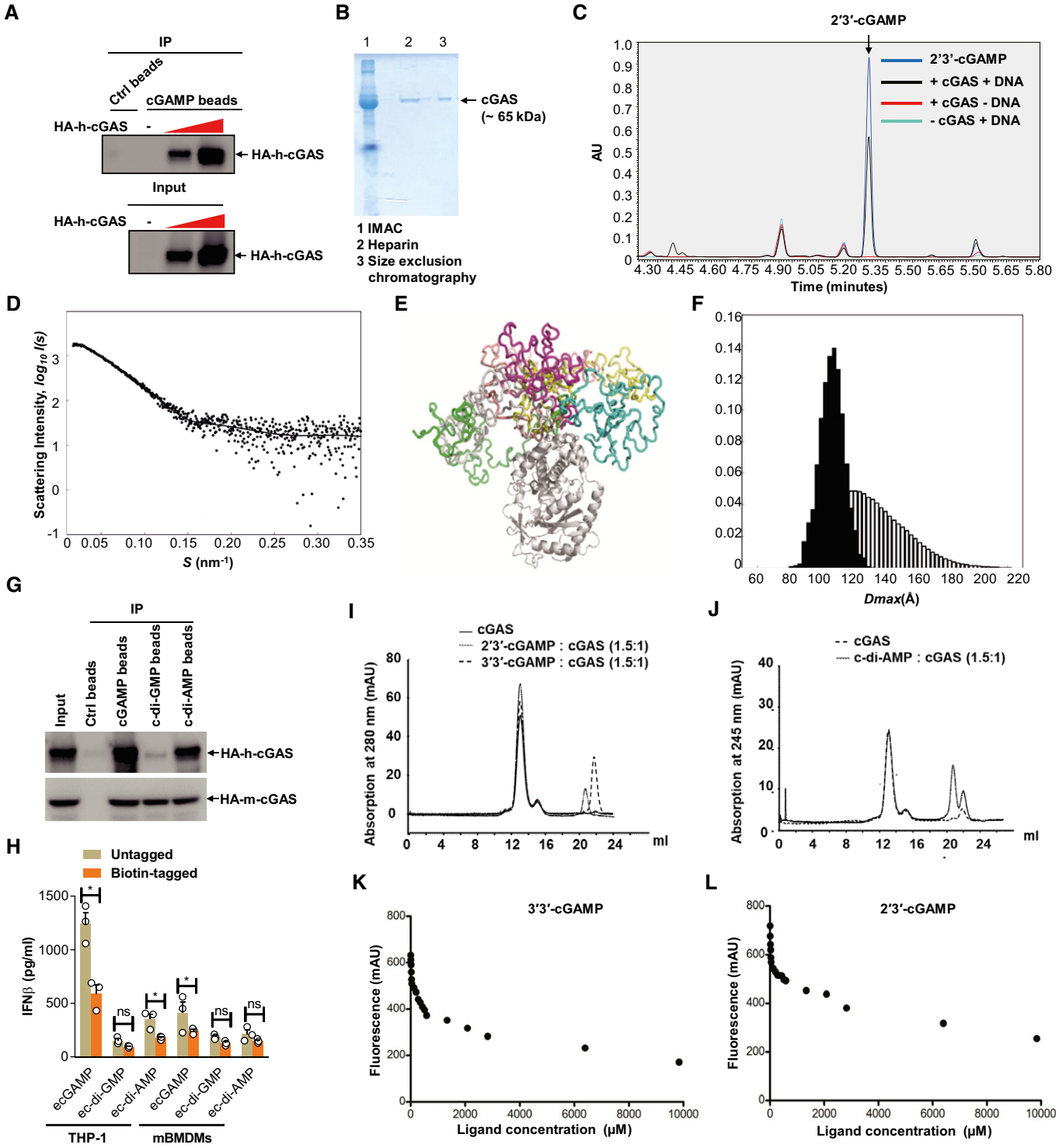

Figure EV3.

◀ **Figure EV3. Purification of cGAS and its binding to CDNs.**

A Lysates of HEK293T cells transfected with increasing amounts of HA-cGAS were precipitated with control beads (Ctrl) or 2′3′-cGAMP beads (cGAMP) and immunoblotted. Data are representative of three independent experiments.

B SDS–PAGE gel analysis of purified cGAS protein by indicated methods. IMAC, immobilized metal affinity chromatography. Data are representative of two independent experiments.

C Enzyme activity of purified cGAS confirmed by UPLC detection of cGAMP on a Waters BEH Amide Column. Data are representative of three independent experiments.

D The small-angle X-ray scattering analysis of the full-length apo-cGAS. The EOM fit of the measured SAXS data. The goodness-of-the fit $\chi^2 = 1.2$.

E Structural alignment of the representative structures from cGAS apo EOM analysis.

F The D_{max} distributions (the maximum distance within a particle) derived from the EOM analysis of the measured SAXS profile (pool—the white histogram; the selected structures—the black histogram). The ensemble average of D_{max} is 109.1 Å.

G Lysates of HEK293T cells transfected with HA-tagged human cGAS (HA-h-cGAS) or mouse cGAS (HA-m-cGAS) were precipitated with Ctrl beads or beads coupled with cGAMP, c-di-GMP, or c-di-AMP followed by immunoblotting.

H ELISA detection of IFNβ release in the supernatant of THP-1 cells stimulated with untagged or biotin-tagged eCDNs (5 μM) including cGAMP, c-di-GMP, and c-di-AMP for indicated times. Data are means + SD averaged from at least three independent experiments performed with technical triplicates, where each symbol represents the mean of technical triplicates. Two-way ANOVA followed by Bonferroni's *post hoc* test was used for statistical analysis, respectively. $*P < 0.05$; ns, not significant.

I, J Elution profiles of analytical size exclusion chromatography (Superdex 200, GE Healthcare, 10/300 GL). Absorption profiles at 280 nm for detection of cGAS (solid lines) and cGAMPs (dashed lines) (I) and at 245 nm for c-di-AMP (J) are shown. Molecular stoichiometric ratios are indicated. Peak maximum at 13 ml (peak 1) corresponds to dimeric cGAS with a molecular mass of about 120 kDa. Despite the presence of free CDNs (Peaks 2–4), the specific interaction (coelution) of cGAS with CDNs was evident in each case by an increased peak intensity of 36% (2′3′-cGAMP), 17% (3′3′-cGAMP), and 2% (c-di-AMP), respectively. mAU, milli absorbance units. Data are representative of three independent experiments.

K, L Fluorometry assay to detect the binding of 3′3′-cGAMP (K) or 2′3′-cGAMP (L) with purified cGAS. Data are representative of at least three independent experiments.

Source data are available online for this figure.

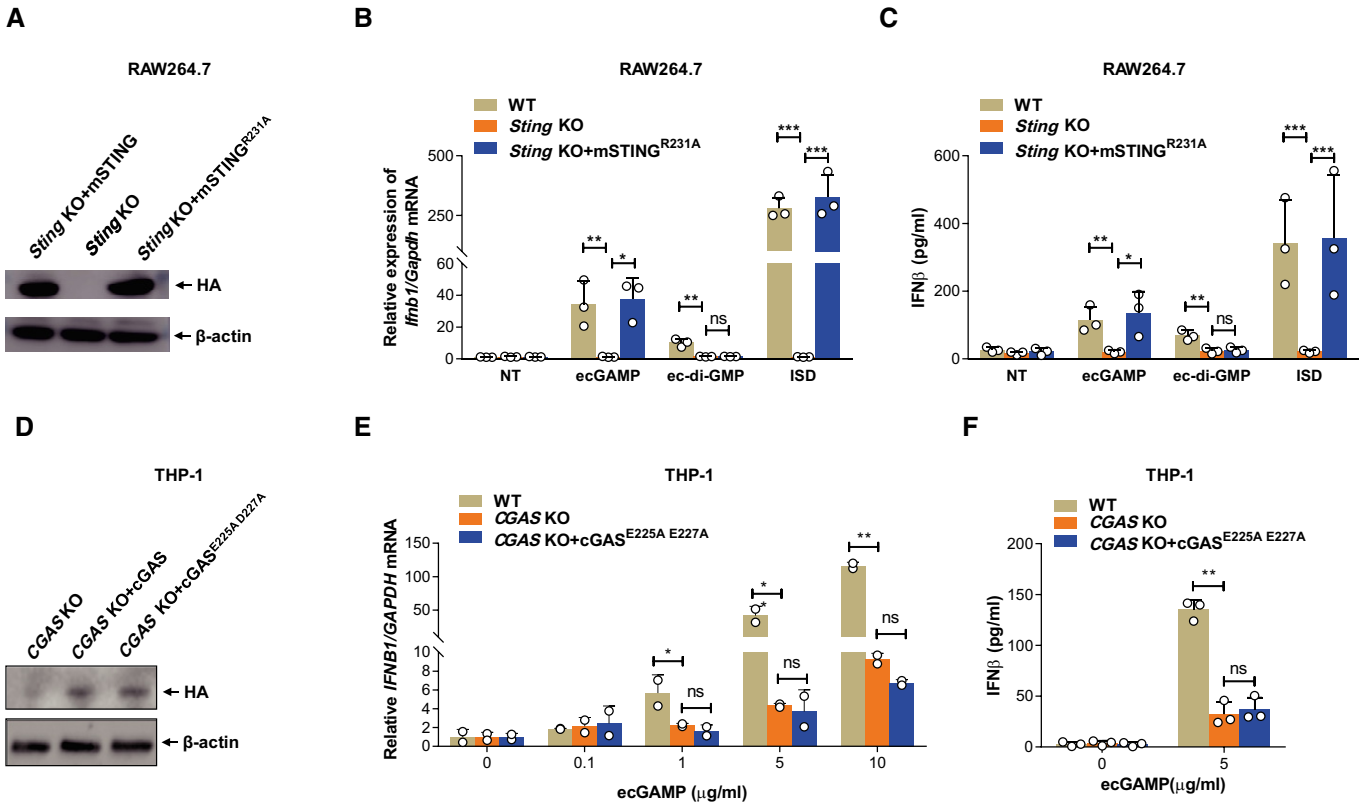

Figure EV4. cGAS facilitates eCDN-induced type I IFN response dispensable of resynthesis of cGAMP by cGAS.

A Western blot detection of indicated proteins in lysates of *Sting* KO RAW264.7 cells (*Sting* KO) and *Sting* KO RAW264.7 cells complemented with WT mouse STING (*Sting* KO+mSTING) or mSTING^{R231A} (*Sting* KO+ mSTING^{R231A}). Data are representative of three independent experiments.

B, C qRT–PCR detection of *Ifnb1* mRNA (B) or ELISA detection of IFNβ in the supernatants (C) of WT, *Sting* KO, and *Sting* KO+ mSTING^{R231A} RAW264.7 cells stimulated with ecGAMP (5 μg/ml) or ec-di-GMP (5 μg/ml) or transfected with ISD.

D Western blot detection of indicated proteins in lysates of *CGAS* KO THP-1 cells (*CGAS* KO) and *CGAS* KO THP-1 cells complemented with WT cGAS (*CGAS* KO+cGAS) or cGAS$^{E225A\ D227A}$ (*CGAS* KO+cGAS$^{E225A\ D227A}$). Data are representative of three independent experiments.

E, F qRT–PCR detection of *IFNB1* mRNA (D) or ELISA detection of IFNβ in the supernatants (E) of WT, *CGAS* KO, and cGAS KO+cGAS$^{E225A\ D227A}$ THP-1 cells stimulated with ecGAMP at indicated concentrations.

Data information: Data (B, C, E, F) are means + SD averaged from at least two independent experiments performed in technical duplicates or triplicate and each symbol represents mean of technical replicates. Two-way ANOVA followed by Bonferroni's *post hoc* test was used for statistical analysis. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.
Source data are available online for this figure.

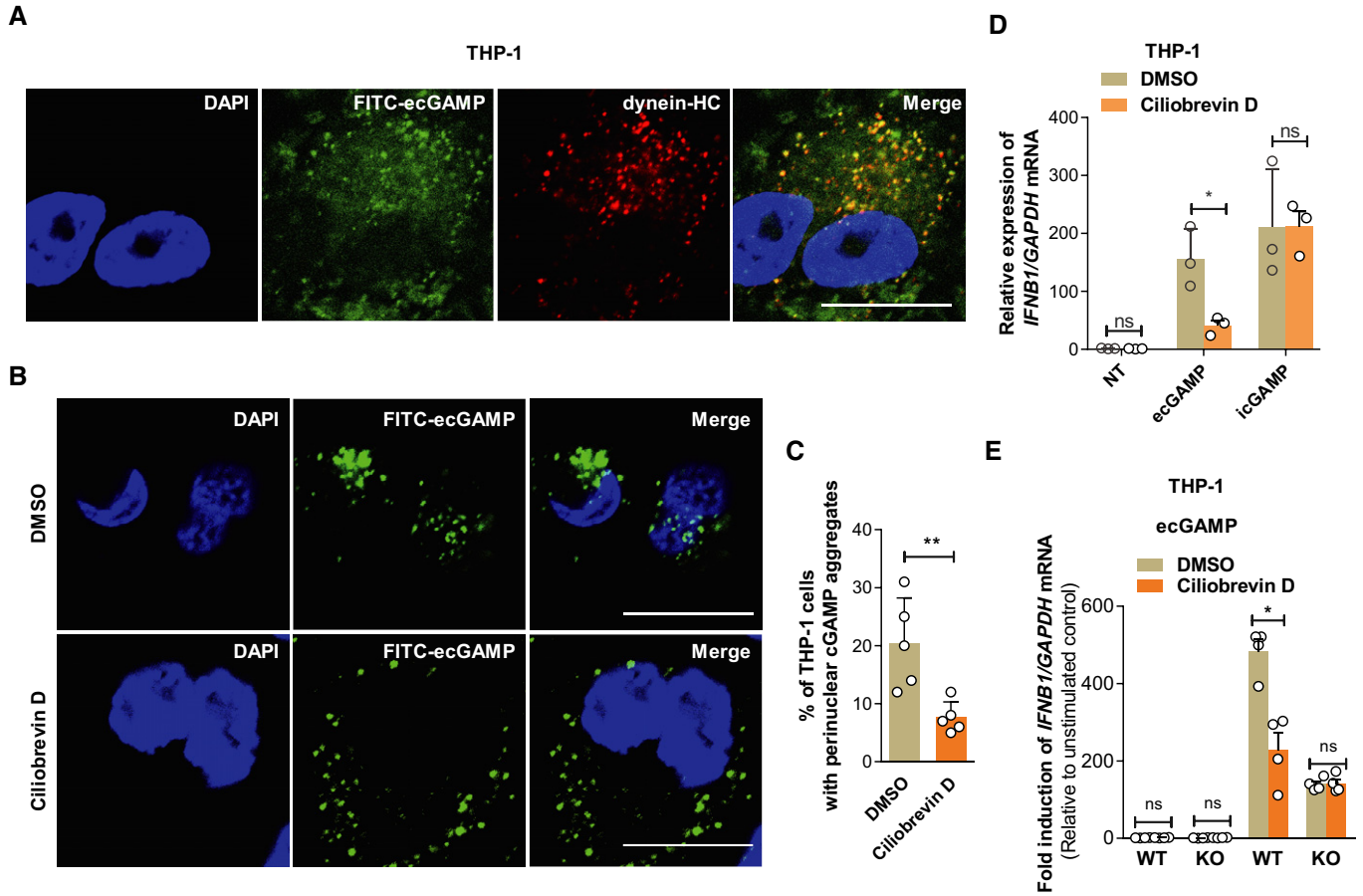

Figure EV5. Dynein contributes to cGAS sensing of eCDNs.

A Immunostaining of dynein heavy chain (HC) (red) in THP-1 cells stimulated with FITC-ecGAMP (5 μg/ml, green) for 2 h, nucleus in blue (DAPI). Data are representative of three independent experiments. Scale bar, 10 μm.

B Cellular localization of ecGAMP in THP-1 cells stimulated with FITC-ecGAMP (5 μg/ml, green) for 2 h in the presence of DMSO or dynein inhibitor ciliobrevin D (50 μM), nucleus in blue (DAPI). Data are presentative of five independent experiments. Scale bar, 10 μm.

C Frequency of perinuclear accumulation of 2′3′-cGAMP in THP-1 cells stimulated with FITC-cGAMP (2 μg/ml) for 2 h in the presence of DMSO or dynein inhibitor ciliobrevin D (50 μM). Data are means + SD averaged from five independent experiments, and approximately 100 cells were imaged and counted in each experiment. Each symbol represents the percentage of THP-1 cells with perinuclear cGAMP aggregates in every independent experiment. Mann–Whitney *U*-test was used for statistical analysis. $^{**}P < 0.01$.

D qRT–PCR detection of *IFNB1* mRNA in THP-1 cells stimulated with ecGAMP (5 μg/ml) or icGAMP (0.1 μg/ml) for 4 h in the presence of DMSO or ciliobrevin D (50 μM).

E qRT–PCR detection of *IFNB1* mRNA abundance in WT and cGAS KO THP-1 cells stimulated with ecGAMP (5 μg/ml) for 4 h in the presence of DMSO or ciliobrevin D (50 μM).

Data information: Data are means + SD (C–E) averaged from at least three independent experiments performed with technical triplicates. Each symbol represents the mean of technical triplicates. Two-way ANOVA followed by Bonferroni's *post hoc* test was used for statistical analysis (C–E). $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant.