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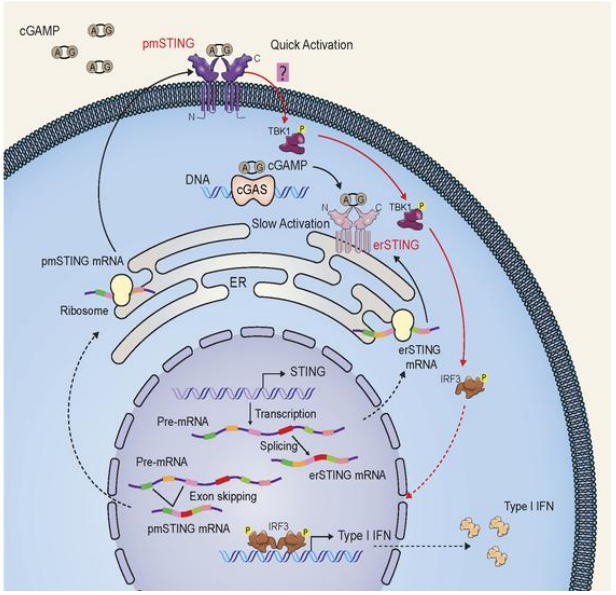

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An alternatively spliced STING isoform localizes in the cytoplasmic membrane and directly senses extracellular cGAMP

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

Abstract

It has been revealed that 2’3’-cyclic-GMP-AMP (cGAMP), a second messenger that activates the antiviral stimulator of interferon genes (STING), elicits an antitumoral immune response. Since cGAMP cannot cross the cell membrane, it is not clear how intracellular STING has been activated by extracellular cGAMP until SLC19A1 was identified as an importer to transport extracellular cGAMP into cytosol. However, SLC19A1 deficient cells also sense extracellular cGAMP, suggesting the presence of mechanisms other than the facilitating transporters for STING sensing extracellular cGAMP. Here, we identified an alternatively spliced STING isoform (plasmatic membrane STING, pmSTING) that localized in the plasma membrane with its C-terminus outside the cell, due to lack of one transmembrane domain in its N-terminus compared to canonical STING, by using immunoprecipitation, immunofluorescence and flow cytometry. Further studies showed that extracellular cGAMP not only promoted the dimerization of pmSTING and interaction of pmSTING with Tank-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), but also enhanced the phosphorylation of TBK1 and IRF3 and production of interferon in pmSTING transfected cells. Additionally, we also identified similar pmSTING isoforms in other species including human. This study suggests a conserved role for pmSTING in sensing extracellular cGAMP and provides insight into cGAMP’s role as an immunotransmitter.

Introduction

Although it was found that DNA can stimulate immune responses as early as 1908 by Mechnikov, the mechanisms regarding to immune response against DNA by immune cells remained unidentified throughout the entire 20th century (1). The identification of stimulator of interferon genes (STING), an endoplasmic reticulum (ER)-resident protein containing four transmembrane domains with its C-terminus projecting into the cytoplasm (2), made a great contribution to the field in 2008. And Dr. Chen's group ultimately explained the detail mechanisms of DNA triggered immune responses in 2013 by demonstrating that cyclic GMP-AMP synthase (cGAS) is the direct cytosolic DNA sensor (3, 4). Once appeared in the cytosol upon infection or genomic damage, the cytosolic DNA binds and activates cGAS, an enzyme that causes the synthesis of 2'3'-cyclic-GMP-AMP (cGAMP) by using GMP and AMP as substrates. cGAMP is a second messenger that binds and activates STING (5-8). Upon ligand binding, homodimerized STING translocates from ER to the perinuclear area wherein it recruits and activates Tank-binding kinase 1 (TBK1), which phosphorylates and activates interferon regulatory factor 3 (IRF3), a transcription factor activating transcription of type I interferons (IFNs) and other immune mediators (9-11).

The cytosolic DNA triggered activation of cGAS-STING-IFN signaling not only plays critical roles in the host defense against microbial infection, but also is critical for the antitumor immune response, and numerous studies have suggested that the activation of STING is a promising strategy to treat cancer (1). It has been shown that extracellular cyclic dinucleotides (CDNs) can activate STING pathway because the administration

of exogenous CDNs displays adjuvant effects and antitumor activity in mice (12), suggesting that extracellular CDNs can activate STING pathway. However, CDNs cannot pass through the lipid bilayer due to their negative charges. How STING senses extracellular CDNs remains elusive. Facilitating mechanisms of CDNs entering cells have been proposed to explain how STING senses extracellular CDNs. Recently, using a genome-wide CRISPR screen, two studies independently identified SLC19A1 as an importer to transport extracellular cGAMP into cytosol. However, SLC19A1 deficient cells also sense extracellular cGAMP (13, 14), suggesting that mechanisms other than the facilitating transporters for STING sensing extracellular CDNs must be examined. Here, we described an alternatively spliced STING isoform embedded within the plasma membrane that activates immune responses by directly sensing extracellular cGAMP.

Results

Extracellular cGAMP activates immune responses in a STING-dependent manner

Although exogenous cGAMP shows antitumor activity in mice, the separate effect of cGAMP on cancer cells and immune cells is not evaluated simultaneously. We showed that extracellular cGAMP does not affect the viability of B16 melanoma cells, whereas cGAMP promotes the antitumor activity of splenocytes in a STING-dependent manner (Figure 1A), which indicates that the antitumor effect of extracellular cGAMP depends on host immune cells. Then we confirmed that extracellular cGAMP directly activates splenocytes (Figure 1B) and various types of immune cells, such as NK cells (Figure

1C), myeloid cells (Figure 1D), T cells and B cells (Supplemental Figure 1A-D), in a STING-dependent manner. Moreover, we demonstrated that extracellular cGAMP, similar to a penetrative STING activator DMXAA, activates the STING pathway evidenced by enhancing the phosphorylation level of TBK1 and IRF3 in splenocytes from WT mice but not STING-deficient mice (*Tmem173*^{gt}) (Figure 1E), and induces the production of IFN-β in a STING-dependent (Figure 1F) and dose-dependent manner (Figure 1G). These results suggest that extracellular cGAMP directly activates immune responses by stimulating the STING pathway.

A cell surface STING projecting its C-terminus outside the cell exists in mouse immune cells

Recently, SLC19A1 has been recognized to import extracellular cGAMP into cytosol. However, SLC19A1 deficient cells also sense extracellular cGAMP (13, 14), which suggests that there are unknown mechanisms by which STING senses extracellular cGAMP. Herein, the topology of transmembrane protein CD38 may provide us a molecular clue to explain this phenomenon. CD38 is a signaling enzyme catalyzing the metabolism of cyclic ADP-ribose (cADPR), an intracellular second messenger regulating cellular Ca^{2+} level. However, its catalytic C-domain locates outside the cell, and binds with extracellular substrates, which induces the internalization of CD38 (15-17). Thus, we suspected whether a STING is on the plasma membrane with its C-terminus outside the cell directly sensing cGAMP. Antibodies against the STING C-terminal domain epitope showed immunoreactivity toward non-permeabilized mouse splenocytes (Figure 2A). The binding of STING antibodies to wild-type (WT)

splenocytes was also confirmed by immunoprecipitation (Figure 2B). Moreover, the co-localization of STING with surface proteins (CD3, CD19 and CD11b) has been observed in WT mice but not in STING-deficient mice (*Tmem173*^{gt}) (Figure 2C and D). Importantly, blocking with antibodies against the STING C-terminus significantly attenuated the production of IFN-β by WT mouse splenocytes in response to extracellular cGAMP (Figure 2E). These results suggest that some STING proteins with their C-terminal cGAMP binding sites outside the cell are expressed on the plasma membrane of mouse immune cells.

An alternatively spliced mouse STING isoform with three transmembrane domains locates in the plasma membrane

In mice, *Tmem173* gene is predicted to encode three alternatively spliced STING isoforms with identical C-terminus but different N-terminus based on the GENE database from NCBI (Gene ID: 72512) (Figure 3A). Thus, an antibody against the C-terminus of STING was used to detect whether all of these predicted STING isoforms were expressed in mice splenocytes, and an additional isoform shorter than the canonical STING was identified (Figure 3B). Additionally, two alternatively spliced STING isoforms were also detected by PCR (Figure 3C) and validated by sequencing (Supplemental Figure 2A) in splenocytes. We also found that both STING isoforms were ubiquitously expressed in different mice tissues (Supplemental Figure 2B and C). Moreover, the C-terminus of the shorter spliced STING isoform (plasmatic membrane STING, pmSTING) was predicted as being outside the cell due to lack of a transmembrane (TM) domain compared with the canonical isoform (endoplasmic

reticulum STING, erSTING) (Figure 3D). To investigate the topology of pmSTING and erSTING, we constructed a pmSTING-GFP (or pmSTING-FLAG) and an erSTING-GFP (or erSTING-FLAG) fusion protein and then expressed them in B16$^{Tmem173-/-}$ cells. We demonstrated that the C-terminus of pmSTING indeed faces outside of the cell by immunoprecipitation (Figure 3E), immunofluorescence (Figure 3F) and flow cytometry (Figure 3G).

Mouse pmSTING isoform directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling

Next, we evaluated the roles of mouse pmSTING in sensing extracellular cGAMP and activating TBK1-IRF3-IFN signaling. A functional study showed that the pmSTING rather than the erSTING isoform specifically senses extracellular cGAMP and induces the production of IFN in B16$^{Tmem173-/-}$ cells that stably express secreted alkaline phosphatase (SEAP) to monitor interferon activity. However, mutation of the phosphorylation site of TBK1 (S316R) in the C-terminus of pmSTING eliminates its capability to induce SEAP production (Figure 4A). Unexpectedly, B16$^{Tmem173-/-}$ cells transfected with erSTING or pmSTING showed obvious elevated SEAP level compared to untransfected cells (Figure 4A). We suspected that endogenous cGAMP due to plasmid DNA triggered activation of cGAS may contribute to the quite high basal levels of SEAP inB16$^{Tmem173-/-}$cells transfected with erSTING or pmSTING. As expected, we confirmed that knockdown of cGAS decreased the basal levels of SEAP and did not affect pmSTING sensing extracellular cGAMP (Supplementary Figure 3A and B). Moreover, we detected the potential roles of SLC19A1 in pmSTING sensing

extracellular cGAMP and showed that SLC19A1 was not necessary for the activation of pmSTING induced by extracellular cGAMP (Supplementary Figure 3C and D). Additionally, we showed that extracellular cGAMP enhanced the phosphorylation of TBK1 and IRF3 (Figure 4B), induced dimerization of pmSTING (Figure 4C and D) and promoted the interaction of pmSTING with TBK1 and IRF3 (Figure 4E) in pmSTING transfected B16$^{Tmem173-/-}$ cells, but failed to observe these results in erSTING transfected B16$^{Tmem173-/-}$ cells (Figure 4C-E). Collectively, these results suggest that pmSTING isoform is expressed at the mouse cell surface with its C-terminus outside the cell and directly senses extracellular cGAMP.

An alternatively spliced isoform of human STING with one transmembrane domain embeds in the plasma membrane and projects its C-terminus outside the cell

Identification of a pmSTING in mice drived us to consider if a pmSTING isoform was also existed in human. We confirmed that a plasmatic membrane STING with C-terminus outside cells exists in human PBMCs using flow cytometry (Figure 5A), immune precipitation (Figure 5B) and immunofluorescence assays (Figure 5C). In addition to encoding the canonical STING isoform (h-erSTING), the human *TMEM173* gene is also predicted to encode an alternative spliced STING isoform with one TM domain based on the GENE database from NCBI (Gene ID: 340061) (Figure 5D and E), and the expression of this transcript has been confirmed in PBMCs by immune blot (Figure 5F) as well as PCR and sequencing (Figure 5G and Supplemental Figure 4). We further demonstrated that this human pmSTING (h-pmSTING) is expressed in the

plasma membrane and that its C-terminus is localized in the extracellular space using immunofluorescence (Figure 5H) and flow cytometry (Figure 5I).

Human pmSTING directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling

Furthermore, we investigated whether such a human pmSTING isoform also contributes to sensing extracellular cGAMP and activating TBK1-IRF3-IFN signaling. We showed that extracellular cGAMP not only induced IFN production (Figure 6A) and enhanced the phosphorylation of TBK1 and IRF3 (Figure 6B) in h-pmSTING rather than h-erSTING transfected 293T cells, but also promoted the dimerization of h-pmSTING (Figure 6C and D) and interaction of h-pmSTING with TBK1 and IRF3 (Figure 6E) in h-pmSTING transfected 293T cells. By comparison, extracellular cGAMP had no effect on the dimerization of h-erSTING (Figure 6C and D) and interaction of h-erSTING with TBK1 and IRF3 (Figure 6E) in h-erSTING transfected 293T cells. These results suggest that pmSTING rather than erSTING directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling in human cells.

Finally, we evaluated if such a pmSTING isoform with its C-terminus outside the cell is conserved across animals. Based on the alternatively spliced isoform sequence, we predicted that the STING isoform with its C-terminus outside the cell also exists in many other species (Figure7A), suggesting that pmSTING may be a conserved membrane molecule sensing extracellular cGAMP in animal kingdom.

Discussion

The canonical STING resides in the ER, thus activating STING requires cytoplasmic localization of its ligands (3, 18, 19). This has inspired several strategies to deliver the CDNs into the cytoplasm either through cell permeabilization or liposome-mediated transfection (20-23). Recently, it was reported that some membrane molecules facilitate transfers of extracellular CDNs into cytosol. For example, upon infection, cGAMP could be transferred from HSV-1 infected cells into bystander cells through LRRC8, a subunit of volume-regulated anion channels (24). In cancer tissues, cGAMP released from dying cells could enter tumor-associated macrophages and activate STING signaling through the ATP-gated channel P2X7R (25). However, it is unknown if LRRC8 and P2X7R facilitate transfers of extracellular cGAMP in other settings. Two groups independently identified that the reduced folate carrier SLC19A1 is a direct cGAMP importer in many cell types. However, SLC19A1 deficient cells also sense extracellular cGAMP (13, 14). Consistently, we showed that knocking down SLC19A1 expression in B16$^{Tmem173-/-}$ cells does not affect pmSTING's ability to sense extracellular cGAMP (Supplemental Figure 3C and D). Collectively, these results suggest that facilitating transporters may not be the only mechanism for STING sensing extracellular CDNs.

We identified an alternatively spliced STING isoform which ubiquitously presents in the plasma membrane of human and mouse cells with its C-terminus extending to the outside of cell surface. More interestingly, similar STING isoform was also identified in other animal species in our study, suggesting a broad and conserved role for pmSTING in sensing extracellular CDNs. Additionally, it was found that the canonical

STING ectopically expressed in STING deficient cells could only be activated by intracellular but not extracellular cGAMP (26). Consistently, we showed that pmSTING rather than erSTING could be activated by extracellular cGAMP in both human and mouse cells, supporting the idea that the pmSTING isoform is the sensor for extracellular cGAMP.

The C-terminus of STING provides the cGAMP binding domain and signaling transduction domains. We showed that pmSTING shares the same C-terminus with the canonical STING but projects its C-terminus outside cells. This membrane topology seems paradoxical to the pmSTING mediating type I IFN response. A similar phenomenon has been observed in CD38, a single transmembrane protein with its catalytic C-domain outside the cell. CD38 can undergo an extensive internalization upon binding extracellular ligands (15-17). Although the mechanism underlying this ligand-induced internalization has not been fully explained, internalization of cell surface CD38 results in a shift of exocellular cADPR metabolism to the cytosol (27). In this study, we showed the essential roles of C-terminus in pmSTING sensing extracellular cGAMP, and found the internalization of pmSTING upon stimulating by extracellular cGAMP (Supplemental Figure 4A and B). However, the molecular mechanism by which extracellular cGAMP activates pmSTING translocation remains unclear. Plasma membrane protein Tspan8 containing four transmembrane domains were identified to translocate from the plasma membrane to the nucleus. Mechanistically, it is not in the form of vesicles but as a Tspan8/cholesterol complex to be translocated into nuclei, and cholesterol is critical for binding and protecting the

hydrophobic transmembrane domains of Tspan8during the translocation process (28), which suggests another mechanism about plasma membrane protein internalization mediated by a non-specific transporter, such as cholesterol. Moreover, the canonical STING translocates from the ER membrane to the perinuclear area to activate TBK1 and IRF3 upon binding cGAMP, and the translocator in ER comprised of TRAPβ, Sec61β and Sec5 (11, 29) contributes to the translocation of STING, while iRhom2 facilitates the assembly of the STING-TRAPβ translocation complex (30). Thus, unknown vector complexes or transporters may contribute to internalization of pmSTING. However, the detail molecular mechanisms of pmSTING internalization triggered by extracellular cGAMP need to be further investigated.

In conclusion, we found an alternatively spliced STING isoform embedded within the plasma membrane with its C-terminus outside the cell directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling (Figure 7B). This study provides insight into cGAMP's role as an immunotransmitter and may contribute to developing useful cancer therapeutics targeting cell surface STING.

Methods

Further information can be found in the Supplemental Methods.

Cell culture

HEK293T cell line was obtained from Cell Culture Center of Chinese Academy of Medical Science, and cultured in DMEM (Gibco, USA) supplemented with 10% fetus bovine serum (FBS) (Gibco) and 0.1% Penicillin-Streptomycin solution (Biosharp, China). B16-Blue™ ISG-KO-STING cell line (Invivogen, USA), B16 cell line (Cell

Culture Center of Chinese Academy of Medical Science, Beijing, China), mouse splenocytes and human PBMCs were cultured in RPMI 1640 (Gibco) added with 10% FBS and 0.1% Penicillin-Streptomycin solution. All cells were maintained in standard culture condition (37℃， 5% CO_2).

Animal models

C57BL/6J WT mice were purchased from the animal center of the second affiliated hospital of Harbin Medical University. STING-deficient mice (*Tmem173^{gt})* generated by a forward genetic mutagenesis screen in C57BL/6J mice using the mutagen N-ethyl-N-nitrosourea were purchased from the Jackson Laboratory (31). All the mice were maintained in the specific pathogen free conditions with access to food and water.

Isolation of splenocyte

Fresh spleens were harvested from WT or STING-deficient mice (*Tmem173^{gt})* and gently crushed by the inner piston of the syringe in sterile PBS (Solarbio, China). Then splenocytes were suspended in PBS and filtrated with the filter mesh (100 μm) and concentrated at 1500 g. The cell precipitation was re-suspended in 1 mL RBC lysis buffer to remove the RBCs for 2 minutes. The cell was re-suspended by PBS after concentration for twice at 1500 g.

Isolation of human peripheral blood mono-nuclear cells (PBMCs)

The blood from healthy volunteers was collected into 5 mL EDTA anti-coagulation tubes and diluted with 6 mL sterile PBS. Then the diluted blood was gently added in the tube containing 5 mL Ficoll-Hypaque solution on the bottom. The tube was centrifuged at 1500 g. After centrifugation, the solution in the tube was divided into

three layers, and the intermediate froggy layer containing PBMCs was transferred to a new tube and underwent another centrifugation at 1500 g. The cell precipitation was re-suspended in 1 mL RBC lysis buffer to remove the RBCs for 2 minutes. After centrifugation at 1500 g, the cell precipitation was re-suspended by PBS.

Flow cytometry

Mouse splenocytes were stimulated by 35 μM of 2',3'-cGAMP (Invivogen, USA) or 35 μM of DMXAA (Selleck Chemicals, TX, USA) for 18 hours. Human PBMCs were stimulated by 35 μM of 2',3'-cGAMP for 18 hours. Cells were collected and centrifuged at 1500 g after stimulation by cGAMP or DMXAA. Then cell precipitations were washed twice by PBS. After adjusting the cell number to 2×10^6, surface staining was performed with the following fluorochrome-conjugated antibodies at 4°C in dark place for 30 minutes: FITC anti-mouse CD3 antibody (17A2), PE anti-mouse CD69 antibody (H1.2F3), FITC anti-human CD3 antibody (HIT3a), PE anti-human CD69 antibody (FN50), FITC anti-mouse CD4 antibody (GK1.5), APC anti-mouse CD8 antibody (53-6.7), FITC anti-mouse CD19 antibody (6D5), PE anti-mouse CD86 antibody (GL-1), FITC anti-mouse CD11b antibody (M1/70), PE/Cyanine7 anti-mouse NK-1.1 antibody (PK136). All these antibodies were purchased from BioLegend. To identify the plasma membrane STING isoform, cells were incubated with the primary antibodies against the C-terminus of STING (19851-1-AP, Proteintech; ab92605, Abcam; NBP2-24683SS, Novus Biologicals), anti-GAPDH antibody (60004-1-Ig, Proteintech, USA) and rabbit IgG isotype antibody (Millipore, USA) at 4°C for 1hour. B16-KO-STING cells transfected with erSTING-Flag or pmSTING-Flag were

stimulated by 35 μM of 2',3'-cGAMP for 18hours, cells were incubated with anti-Flag antibody (14793S, Cell Signaling Technology, USA) at 4°C for 1 hour. After washed twice by PBS, cells were stained by the FITC-conjugated secondary antibody (SA00003-2, Proteintech, USA) at room temperature in dark place for 1 hour.

After staining, cells were washed twice by PBS (1500 g, 5minutes) twice and re-suspended with 500 μL PBS and filtrated with the filter mesh (50 μm). All samples were examined by BD LSR Fortessa and analyzed by FlowJo software (TreeStar, Inc., Ashland, OR, USA).

In vitro killing assay

Melanoma B16 cells were seeded into the 96-well plate by 2000 cells per well. They were co-cultured with or without splenocytes (the ratio of splenocytes and B16 cells was 50:1) and treated with 35 μM of cGAMP or vehicle control for 48 hours. 20 μL of 0.5% MTT solution (Solarbio, China) was added into each well of the plate and incubated at 37℃ in dark place for 4 hours. The supernatant was gently replaced with 150 μL of DMSO, and the plate was shaked for 10 minutes. The cell viability was reflected by the absorption at 490 nm detected by a Spectra Max® M5 Multi-Mode Microplate Reader.

ELISA

Mouse splenocytes freshly isolated from two-month-old WT or STING-deficient mice (*Tmem173*^{gt}) were incubated in the 96-well plate at the concentration of 2×10^6/well. After stimulated with 2',3'-cGAMP, c-di-GMP (Invivogen, USA) or DMXAA for 24 hours, the supernatants of splenocytes were collected. In the STING C-terminus

blocking assay, the mouse splenocytes were incubated separately with 10 μg/mL of different antibodies against C-terminus of STING (19851-1-AP, Proteintech; ab92605, Abcam; NBP2-24683SS, Novus Biologicals) at 37°C for 2 hours before stimulating with cGAMP for 24 hours. The interferon β (IFN-β) in the supernatants of splenocytes were detected with Mouse IFN-β ELISA Kit (42400-1, R&D Systems Company, USA), following the manufacturers' protocol.

Western blot

Total proteins were extracted from the collected cell pellets using RIPA buffer (Beyotime Institute of Biotechnology, China) added with protease inhibitor PMSF (Roche, USA) and phosphatase inhibitors (Roche). Proteins were quantified using a BCA Kit (Beyotime Institute of Biotechnology). Equal protein was separated on an SDS-PAGE gel by electrophoresis and transferred onto a PVDF membrane (Millipore). After blocked with 5% BSA at room temperature for 1hour, membranes were incubated with the following primary antibodies: anti-STING rabbit polyclonal antibody (19851-1-AP, Proteintech) diluted at 1: 600, anti-mouse TBK1 antibody (ab40676, Abcam) diluted at 1: 1000, anti- mouse phosphorylated TBK1 (Ser172) antibody (ab109272, Abcam) diluted at 1: 500, anti- mouse IRF3 rabbit polyclonal antibody (11312-1-AP, Proteintech) diluted at 1:1000, anti- mouse phosphorylated IRF-3 (Ser396) rabbit mAb (29047, Cell signaling technology) diluted at 1:1000, anti-mouse cGAS antibody (ab252416, Abcam) diluted at 1:1000, anti-mouse SLC19A1 antibody (25958-1-AP, Proteintech) diluted at 1:1000, anti-GAPDH mouse mAb (60004-1-Ig, Proteintech) diluted at 1:1000. After washing, the membranes were incubated with peroxidase

conjugated secondary antibody (SA00001-1 and SA00001-2, Proteintech, diluted at 1: 2000) for 1hour at 37°C. The ECL system and Bio-RAD Gel Doc XR+ system was used to visualize the blots. All assays were replicated three times.

Immunoprecipitation

Mouse splenocytes or human PBMCs were incubated with 5 μg/mL primary antibodies (rabbit anti STING antibody (ab92605, Abcam; 19851-1-AP, Proteintech; NBP2-24683, Novus, USA), rabbit anti GAPDH antibody (60004-1-Ig, Proteintech), rabbit IgG isotype antibody (A0418, Sigma-Aldrich, USA) at 4°C for 1 hour. The cell pellets were collected by centrifugation at 8000 r/min for 5 minutes and then were lysized with RIPA buffer with PMSF on ice for 10 minutes. The HRP-conjugated goat anti-rabbit IgG (SA00001-2, Proteintech) (1:2000) was used to detect the rabbit IgG antibodies binding on the cell membrane.

Coimmunoprecipitation

B16-Blue™ ISG-KO-STING cells were co-transfected with pmSTING-Flag and pmSTING-GFP or co-transfected with erSTING-Flag and erSTING-GFP by using jetPRIME transfection regent (Polyplus Transfection, NY, USA) according to manufacturer's instruction. 24 hours after transfection, cells were treated with or without cGAMP (35 μM) for 4 hours, and then harvested and lysed on ice using pre-chilled IP lysis buffer with 1×Protease inhibitor for 30 minutes. The supernatant was collected by centrifugation at 4°C, 10,000 g for 20 minutes. Add 4 μg anti-Flag antibody (T0003, Affinity, USA) or anti-GFP antibody (50430-2-AP, Proteintech, USA) or same amount of IgG to 500 μL of lysate containing 3 mg total protein in spin columns and

incubated overnight at 4°C.And then add 50 μL of resuspended Protein A sepharose beads slurry to the spin columns and incubated at 4°C for 4 hours to precipitate the immune complex. Remove the supernatant naturally, and wash the precipitated complex 5 times with 800 μL 1×washing buffer (containing 1×Protease inhibitor). Put the spin columns into a new 1.5 ml EP tube and elute the precipitated complex with 40 μL elution buffer by centrifugation at 10,000 rpm for 1 minute. At last, add 10 μL Alkali neutralization buffer and 5×sample buffer to eluted solutions. The immunoprecipitated GFP or Flag was detected by western blot.

PCR

Total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used to synthesize cDNA using Reverse Transcriptase M-MLV (RNase H) (H2640A, TAKARA, Japan) following the manufacturers' protocol.

The PCR reaction mixture contains 1 μL of cDNA, 10 μL of 2×Pfu Mix (Sciencestar, China), 2 μL of primers and 7 μL of nuclease-free water underwent 35 PCR cycles consisting of denaturation for 10 seconds at 94℃, annealing for 30 seconds at 60℃ and prolongation for 60 seconds at 72℃. All primers used in PCR were synthesized by Genewiz. Primers for detecting STING isoforms were included in supplementary table 1.

Plasmid construction

The DNA sequence encoding mouse or human pmSTING ORF fused EGFP or 3× FLAG, mouse or human erSTING ORF fused EGFP or 3×FLAG and sequence

encoding mouse pmSTING ORF with mutation of TBK1 phosphorylation site (S316R) fused EGFP were synthesized de novo respectively, and then cloned into pcDNA3.1 vector.

Lucia and SEAP reporter assay

HEK293T cells or B16-Blue™ ISG-KO-STING cells were seeded into 24-well plates at 2×10^5/well for 24 hours. HEK293T cells were transfected with 400 ng of h-pmSTING or h-erSTING plasmid and 800 ng of IFN promoter luciferase reporter plasmid IFNβ-pGL3 (Addgene, USA) by using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. B16-Blue™ ISG-KO-STING cells were transfected with or without si-cGAS or si-SLC19A before transfection with 2 μg of pmSTING or erSTING plasmids by using Lipofectamine 2000. Six hours after transfection, medium was replaced by serum-free medium with or without cGAMP (35 μM). After treatment for 8hours by cGAMP, 20 μL of supernatant from B16-Blue™ ISG-KO-STING cells and 180 μL of alkaline phosphatase (SEAP) substrate (Invivogen) were added into 96-well plate. Then, the plate was incubated at 37℃ for 5 hours. The absorption at 630 nm was detected on a Spectra Max® M5 Multi-Mode Microplate Reader. For HEK293T cells, 10 μL of supernatant was mixed with substrate luciferin (Invivogen) in a 1.5 mL EP tube and the result was immediately read by a Promega Glomax 20/20. All assays were replicated three times.

Immunofluorescent staining and confocal laser scanning microscopy

The mouse splenocytes smear samples and human PBMCs smear samples were fixed by 2% cool paraformaldehyde (PFA) for 5 minutes and blocked by 1% bovine serum

alanine (BSA) (Biofrog, China) for 30 minutes. After washed by cold PBS twice, the staining was performed with the primary antibody cocktail containing rabbit anti-*TMEM*173 antibody (ab189430, Abcam,1:200) and fluorochrome-conjugated antibodies against cellular surface markers of different immune cell types (Biolegend) at 4°C in dark place for 30 minutes. After washed by cold PBST three times, the smear samples were stained with Alex Fluo 594-conjugated goat anti rabbit IgG (Proteintech, 1:100) at room temperature in dark place for 1hour. The nuclear staining was performed by DAPI (Solarbio) at room temperature for 10 minutes. Finally, the smear samples were washed by cold PBST and then sealed with sealing agent (Sigma).

FLAG, erSTING-FLAG or pmSTING-FLAG transfected B16-Blue™ ISG-KO-STING cells or HEK293T cells were fixed by 2% PFA for 5minutes and blocked by 1% BSA for 30 minutes. The slides were incubated by primary antibody against Flag (T0053, Affinity, USA, 1:50) at 4℃ overnight. Then the slides were stained with WGA Lectin (FITC) (GTX01502, GeneTex, Irvine CA, 1:500) for 15 minutes at room temperature in dark place. After washed by cold PBS three times, the slides were stained with Alex Fluo 594-conjugated goat anti rabbit IgG (Proteintech, 1:100) at room temperature in dark place for 1hour. Finally, the slides were stained with DAPI and sealed with 50% glycerin as mentioned above. Images were captured by Nikon C2 confocal microscope.

Statistics

Statistical analysis and graph generation were performed with GraphPad Prism 6 (San Diego, CA, USA). All data were presented as the mean ± SD. A 2-tailed Student's *t* test was used to compare 2 groups of independent samples in luciferase reporter assays. A

two-way ANOVA was used to determine the variation among or between groups when analyzing the data of ELISA. A one-way ANOVA and Dunnett's test were used to determine the variation of cGAMP induced INF-β production among different antibodies pretreated splenocytes detected by ELISA. A *p* value of less than 0.05 was considered statistically significant.

Study approval

All animal experiments were performed in accordance with protocols approved by the Harbin Medical University Research Ethics Committee.

Author contributions

X.L. and T.Z. designed experiments and wrote the paper with input from all authors. Y.Z. performed experiments and analyzed the data. X.Z. and X.A. performed experiments. M.W. and J.S. prepared the figures. S.W., C.L., and S.L. assisted with data acquisition and analysis. X.L. and T.Z. supervised the project.

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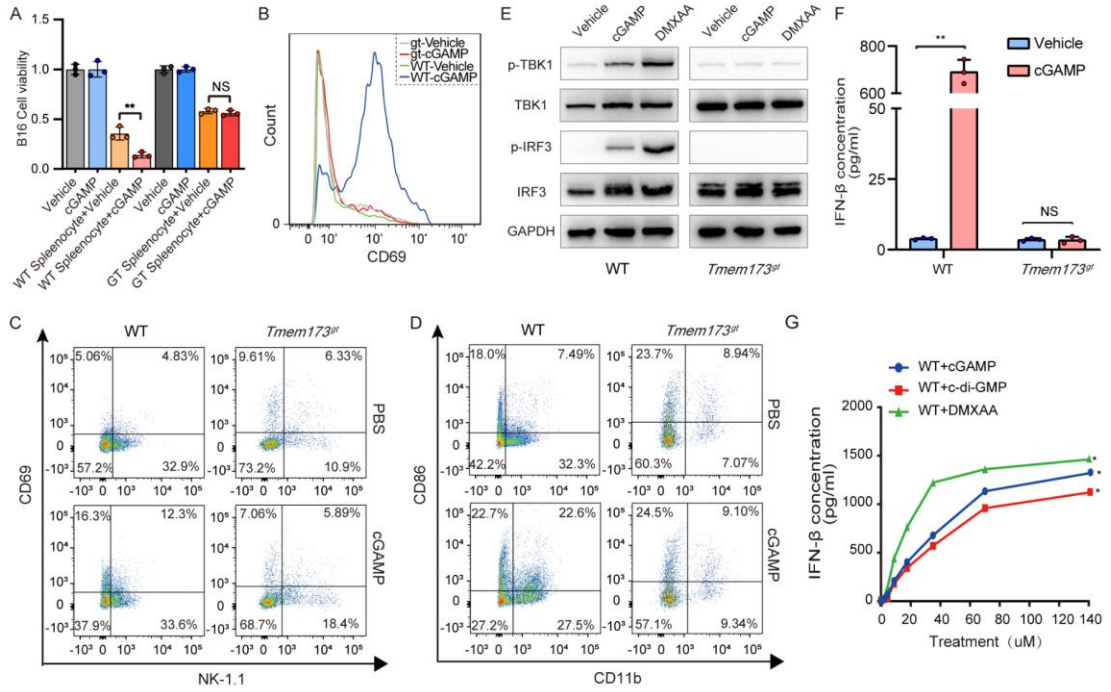

Figure 1. Extracellular cGAMP activates immune responses in a STING-dependent manner. (A) The impact of cGAMP on the viability of B16 cells co-cultured with or without splenocytes from WT or STING-deficient mice (*Tmem173*^{gt}) was detected by MTT assay (n=3). ** represents $p<0.01$, NS means not significant, by 2-tailed, paired Student's *t* test. (B) The expression of CD69 was detected by flow cytometry in WT or *Tmem173*^{gt} splenocytes treated with vehicle or cGAMP. (C) The expression of CD69 was detected by flow cytometry in WT or *Tmem173*^{gt} NK cells treated with vehicle or cGAMP. (D) The expression of CD86 was detected by flow cytometry in WT or *Tmem173*^{gt} myeloid cells treated with vehicle or cGAMP. (E) The phosphorylated TBK1 and phosphorylated IRF3 were detected by Western blot in WT or *Tmem173*^{gt} splenocytes treated with vehicle, cGAMP, or DMXAA respectively. (F) The production of IFN-β in WT or *Tmem173*^{gt} splenocytes treated with vehicle or cGAMP was detected by ELISA (n=3). ** represents $p<0.01$, NS means not significant, by 2-tailed, paired Student's *t* test. (G) The production of IFN-β in WT splenocytes treated with different concentrations of cGAMP, c-di-GMP, or DMXAA was detected by ELISA (n=3). * represents $p<0.05$, by a two-way ANOVA analysis, data were presented as the mean ± SD.

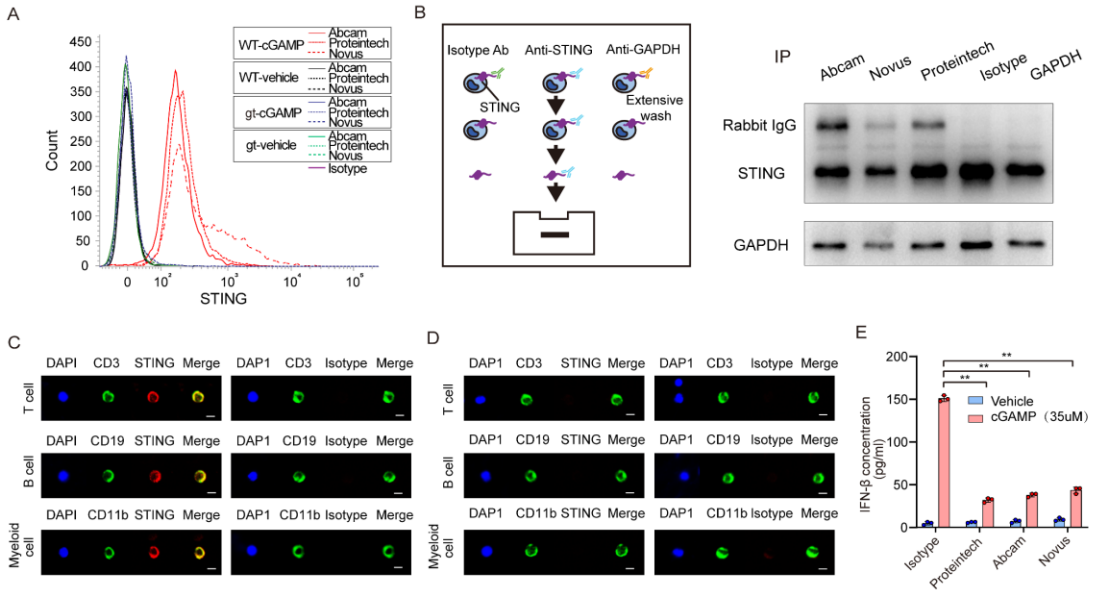

Figure 2. Identification of a cell surface STING projecting its C-terminus outside cells in splenocytes. (A) A cell surface STING with its C-terminus outside mouse splenocytes was detected by three antibodies against STING C-terminal epitope using flow cytometry. (B) WT splenocytes were incubated with indicated antibodies, and then washed and lysized. Immune blot was performed to detect the existence of IgG in the cell lysate by using a second antibody against rabbit IgG. (C) Co-localization of cell surface STING with surface protein of T cells (CD3), B cells (CD19) and myeloid cells (CD11b) from C57BL/6 mice were detected by using confocal microscopy. Scale bars: 5 μm. (D) Detecting the expression of cell surface STING and surface protein of T cells (CD3), B cells (CD19) and myeloid cells (CD11b) from STING-deficient mice (*Tmem173*^{gt}) by using confocal microscopy. Scale bars: 5 μm. (E) Extracellular cGAMP induced production of IFN-β was detected by ELISA in WT splenocytes pre-incubated with indicated antibodies (n=3). ** represents $p<0.01$, by one-way ANOVA and Dunnett's test for the comparison with isotype antibody and cGAMP treatment group.

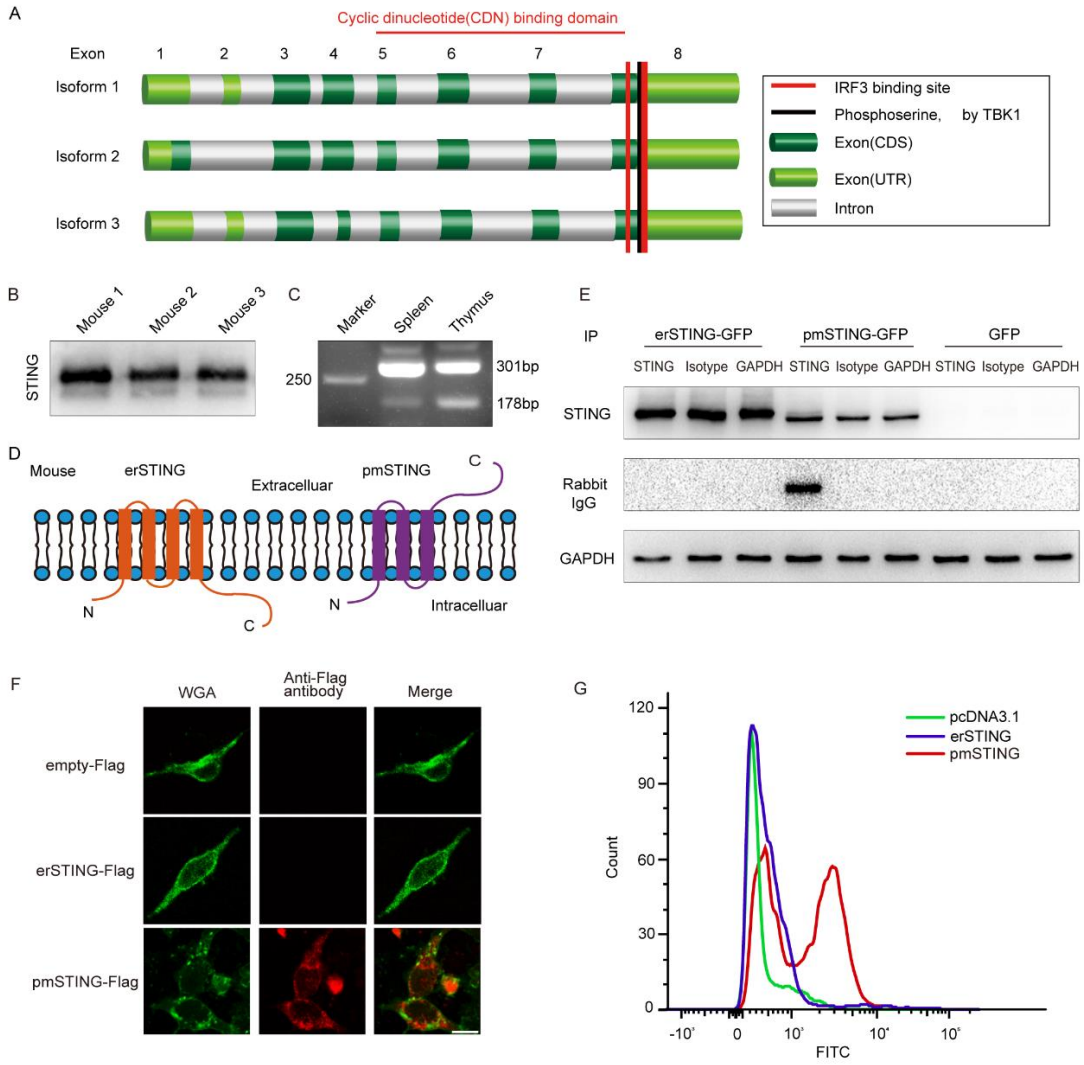

Figure 3. An alternatively spliced STING isoform with three TM domains locates in the plasma membrane of mouse splenocytes. (A) The predicted exon structure and the schematic of functional domains in C-terminus of mouse *Tmem173* transcript variants based on the GENE database from NCBI. (B) Different STING isoforms were detected by immune blot in splenocytes from three independent C57BL/6 mice. (C) Two STING isoforms with different N-terminus were detected by RT-PCR in mouse spleen and thymus. (D) Predicted plasma membrane topology of detected mouse STING isoforms. (E) B16$^{Tmem173\text{-}/\text{-}}$ cells transfected with erSTING-EGFP, pmSTING-EGFP or EGFP were incubated with indicated antibodies, and then washed and lysized. Immune blot was performed to detect the existence of IgG in the cell lysate by using a second antibody against rabbit IgG. (F-G) B16$^{Tmem173\text{-}/\text{-}}$ cells were transfected with erSTING-FLAG, pmSTING-FLAG or vector plasmid respectively. An antibody against FLAG has been used to detect FLAG projecting outside cells by using immunofluorescence (F) and flow cytometry (G), respectively. Scale bars: 20 μm.

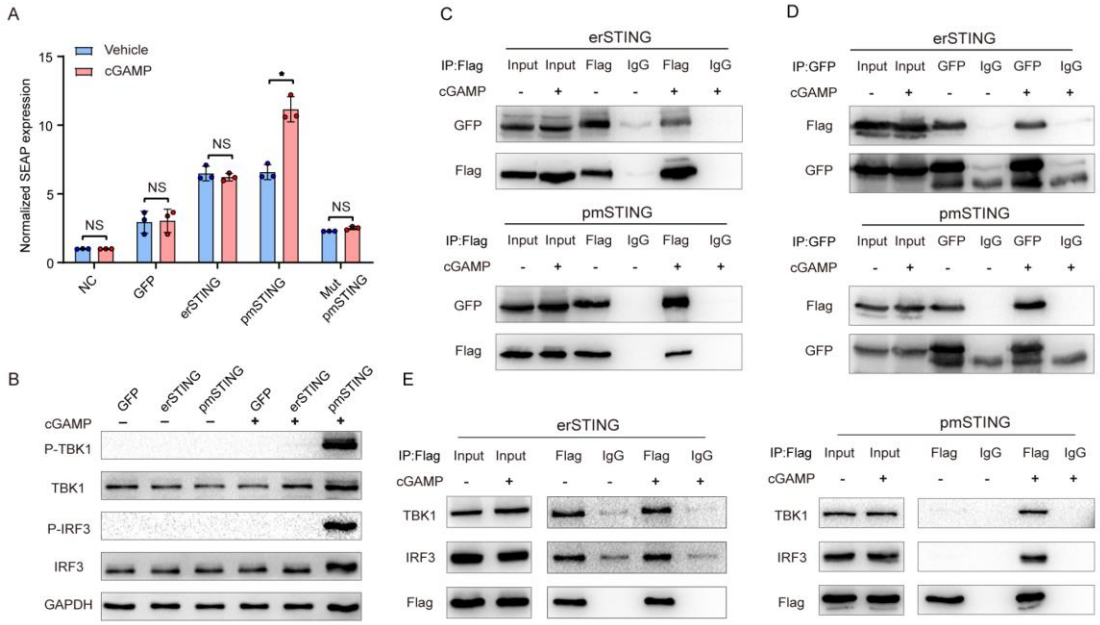

Figure 4. Mouse pmSTING isoform directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling. (A) B16$^{Tmem173-/-}$ cells stably expressing alkaline phosphatase to monitor interferon activity were transfected with erSTING-FLAG, pmSTING-FLAG or mu-pmSTING-FLAG with mutation in TBK1 phosphorylation site (S316R). The alkaline phosphatase was detected in these cells after treated with vehicle or cGAMP (n=3). * represents p<0.05, NS means not significant, by 2-tailed, paired Student's t test. (B) The phosphorylation level of TBK1 and IRF3 has been detected in B16$^{Tmem173-/-}$ cells transfected with erSTING-FLAG or pmSTING-FLAG upon treatment with vehicle or cGAMP using western blot. (C-D) B16$^{Tmem173-/-}$ cells were transfected with both erSTING-FLAG and erSTING-EGFP or both pmSTING-FLAG and pmSTING-EGFP, and then treated with vehicle or cGAMP. Immunoprecipitation using anti-FLAG antibody (C) or anti-GFP antibody (D) was performed to detect the dimerization of pmSTING or erSTING in response to extracellular cGAMP. (E) B16$^{Tmem173-/-}$ cells were transfected with erSTING-FLAG or pmSTING-FLAG and then treated with vehicle or cGAMP. Immunoprecipitation using anti-FLAG antibody was performed to detect the interaction between pmSTING (or erSTING) and TBK1 or IRF3 respectively.

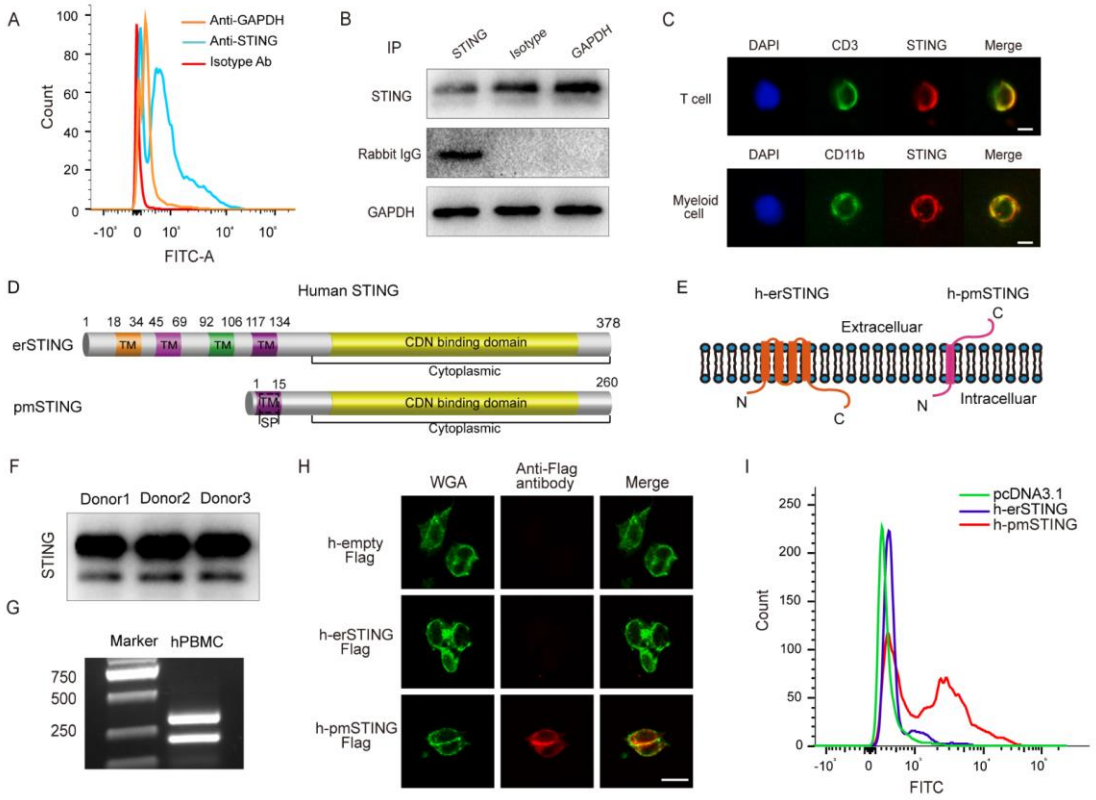

Figure 5. An alternatively spliced isoform of human STING locates in the plasma membrane. (A) Flow cytometry was used to detect the cell surface STING with its C-terminus outside human PBMCs. (B) Human PBMCs were incubated with indicated antibodies, and then washed and lysized. Immune blot was performed to detect the existence of IgG in the cell lysate by using a second antibody against rabbit IgG. (C) Co-localization of cell surface STING with surface protein of T cells (CD3) and myeloid cells (CD11b) from human PBMCs were detected by using confocal microscopy. Scale bars: 10 μm. (D) Exon structure of predicted human *TMEM173* transcript variants based on the GENE database from NCBI. (E) Predicted plasma membrane topology of STING isoforms in homo sapiens. (F) Two STING isoforms were detected by an antibody against STING C-terminal epitope using immune blot in human PBMCs from three healthy donors. (G) Two STING isoforms with different N-terminus were detected by RT-PCR in human PBMCs. (H-I) 293T cells were transfected with h-erSTING-FLAG, h-pmSTING-FLAG or vector plasmid respectively. An antibody against FLAG was used to detect FLAG projecting outside cells by using immunofluorescence (H) and flow cytometry (I), respectively. Scale bars: 20 μm.

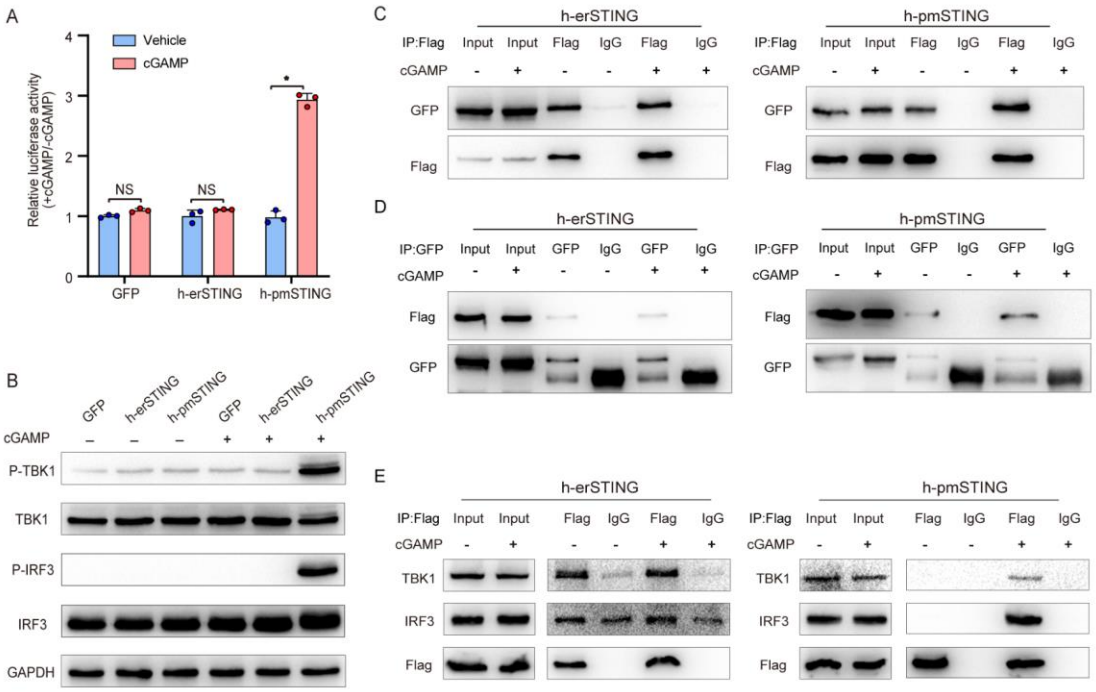

Figure 6. Human pmSTING isoform also directly senses extracellular cGAMP and activates TBK1-IRF3-IFN signaling. (A) 293T cells were co-transfected h-erSTING-FLAG or h-pmSTING-FLAG with luciferase reporter sensing IFN production. The luciferase activity was detected in these cells after treated with vehicle or cGAMP (n=3). * represents $p<0.05$; NS means not significant, by 2-tailed, paired Student's *t* test. (B) Phosphorylation level of TBK1 and IRF3 were detected in 293T cells transfected with h-erSTING-FLAG or h-pmSTING-FLAG upon treatment with vehicle or cGAMP using western blot. (C-D) 293T cells were transfected with both h-erSTING-FLAG and h-erSTING-EGFP or both h-pmSTING-FLAG and h-pmSTING-EGFP, and then treated with vehicle or cGAMP. Immunoprecipitation using anti-FLAG antibody (C) or anti-GFP antibody (D) was performed to detect the dimerization of h-pmSTING or h-erSTING in response to extracellular cGAMP. (E) 293T cells were transfected with h-erSTING-FLAG or h-pmSTING-FLAG and then treated with vehicle or cGAMP. Immunoprecipitation using anti-FLAG antibody was performed to detect the interaction between h-pmSTING (or h-erSTING) and TBK1 or IRF3 respectively.

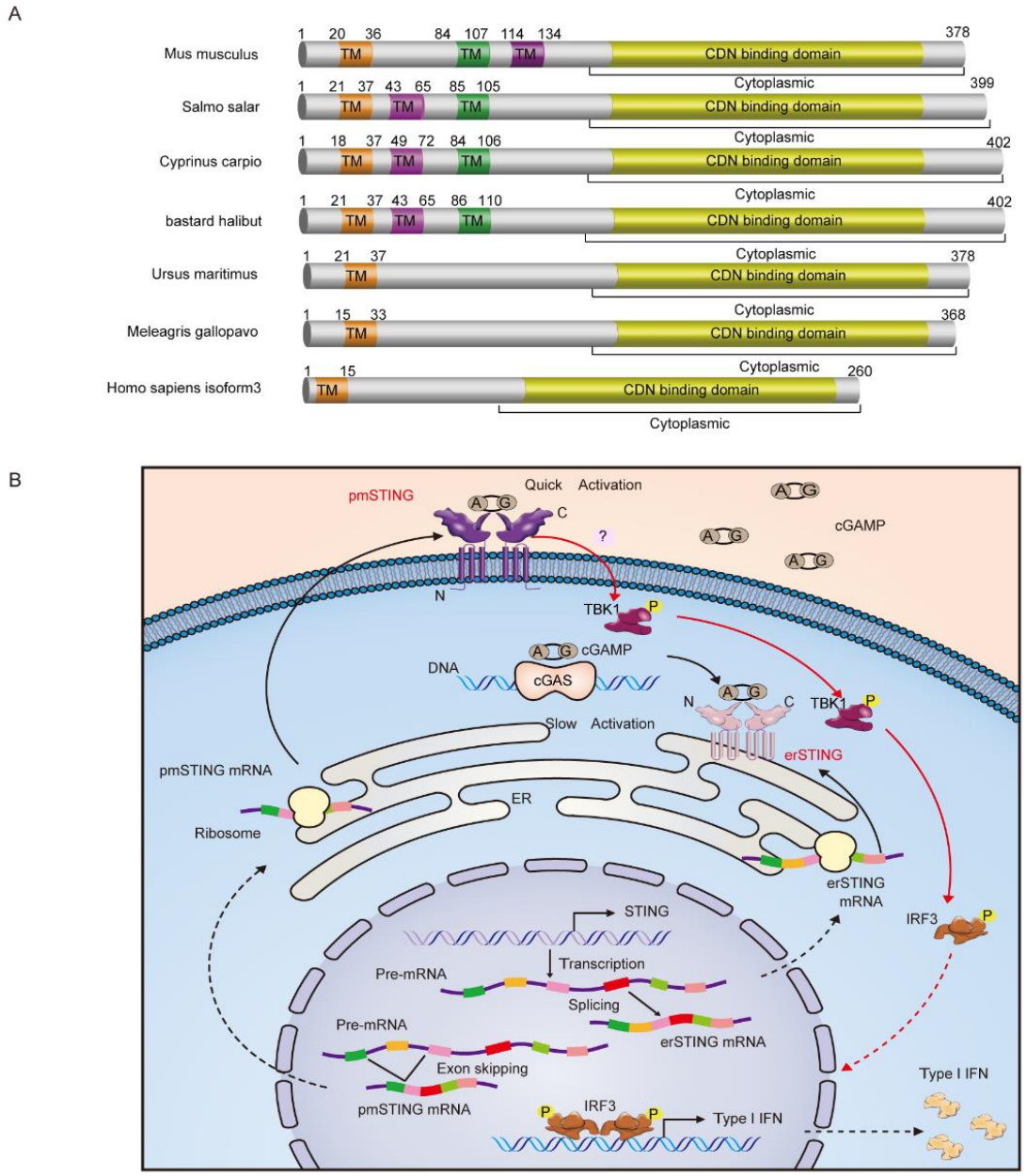

Figure 7. The schematic of predicted pmSTING in various species and the model of pmSTING sensing extracellular cGAMP and prompting IFN production. (A) Multiple protein sequence alignment of STING proteins orthologous to mouse pmSTING isoform. The STING proteins in different species showed here meet the following criteria: 1) possess an odd number of predicted TM domains; 2) possess a C-terminal domain identical to the respective species' canonical STING protein. (B) A model of the generation of two alternatively spliced STING isoforms and how they sense extracellular and intracellular cGAMP respectively. Two STING transcripts generated by alternatively splicing are translated in the cytoplasm and transported to the plasmatic membrane (pmSTING) and the endoplasmic reticulum (erSTING) respectively. Upon binding the intracellular cGAMP synthesized by cytosolic DNA

activated cGAS, erSTING undergoes homodimerization and translocates from ER to the perinuclear area wherein it recruits and activates TBK1, which phosphorylates transcription factor IRF3 and results in translocation of IRF3 from cytoplasm to the nuclear to induce transcription of interferon and other immune cytokines. Whereas the extracellular cGAMP released by dead cells directly binds pmSTING and causes homodimerization and translocation of pmSTING from plasmatic membrane to the perinuclear area wherein it activates TBK1-IRF3-IFN signaling.