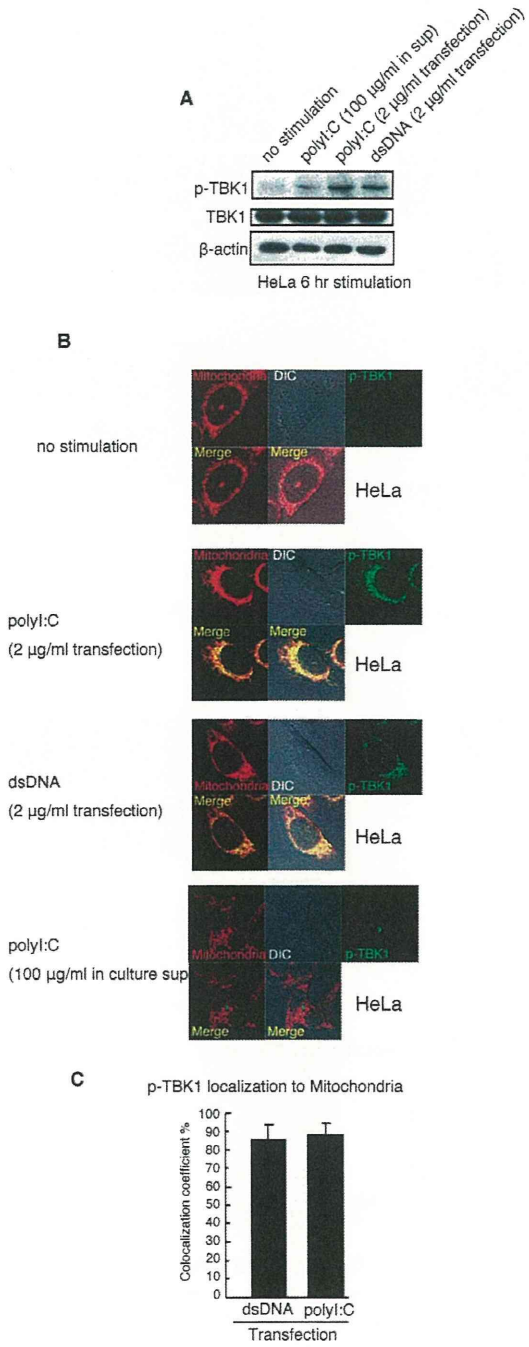

Figure 2. Mitochondrial localization of p-TBK1 in response to cytoplasmic nucleic acids in HeLa cells. (A) HeLa cells were stimulated with 100 μg/ml of polyI:C (no transfection), 2 μg/ml of polyI:C (by transfection) or 2 μg/ml of salmon sperm dsDNA (by transfection) in 6-well plate. At 6 h after stimulation, cell lysates were prepared and subjected to SDS-PAGE. Proteins were detected by western blotting using anti-TBK1, p-TBK1, and β-actin antibodies.

(B) HeLa cells were stimulated with 100 μg/ml of polyI:C (no transfection), 2 μg/ml of polyI:C (by transfection), or 2 μg/ml of salmon sperm dsDNA (by transfection) in 24-well plate. At 6 h after stimulation, cells were fixed and stained with anti p-TBK1 antibody and Mitotracker Red.

(C) Colocalization coefficients of p-TBK1 with mitochondria were determined (mean ±sd, n = 3).

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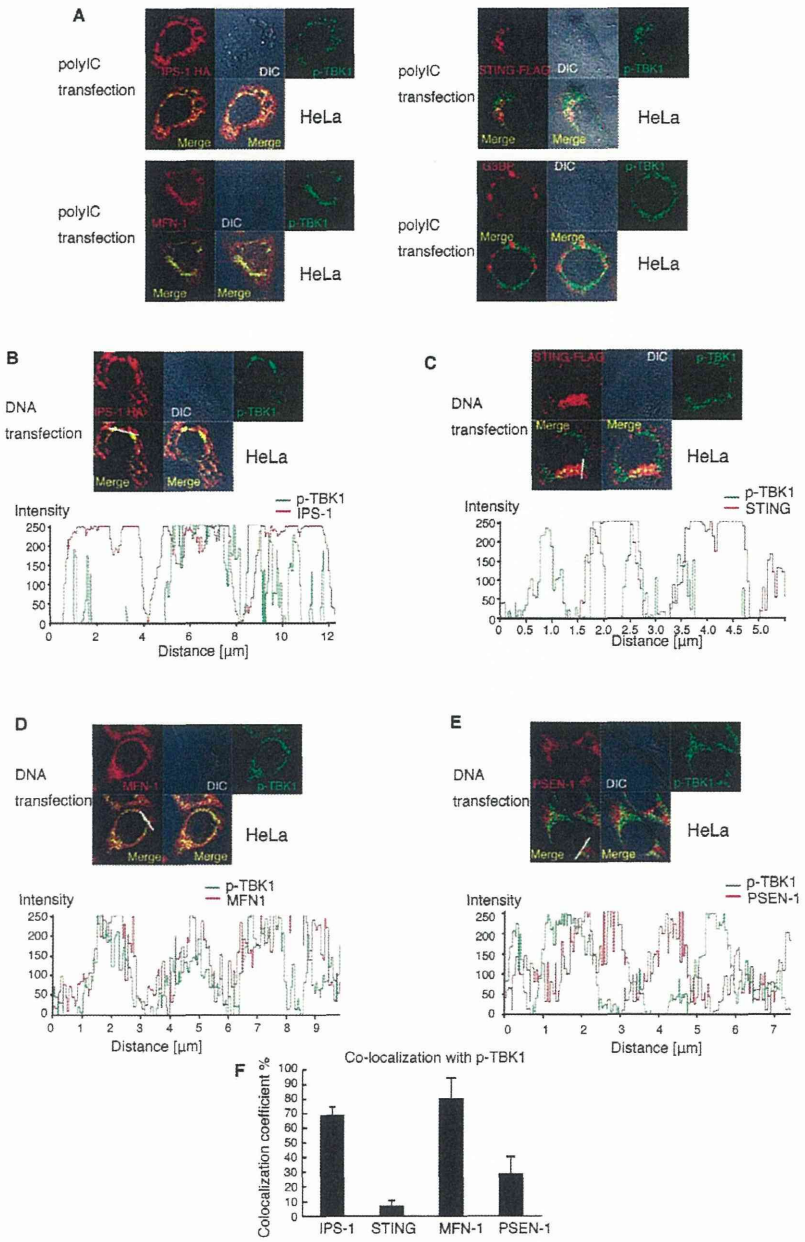

Figure 3. Co-localization of p-TBK1 with IPS-1 in HeLa cells. (A) HeLa cells were transfected with 1 μg of polyI:C in 24-well plate. At 6 h after transfection, cells were fixed and stained with anti-HA, p-TBK1, FLAG, MFN-1, and/or G3BP antibodies. To observe IPS-1 and STING localization, HeLa cells were transfected with HA-tagged IPS-1 or FLAG-tagged STING expression vectors 24 h before stimulation.

(B and C) HeLa cells were transfected with 0.3 μg of HA-tagged IPS-1 (B) or FLAG-tagged STING (C) expression vectors. At 24 h after transfection, HeLa cells were stimulated with 1 μg of salmon sperm dsDNA by transfection in 24-well plate. At 6 h after stimulation, cells were fixed and stained with anti-p-TBK1 and anti-HA or FLAG antibodies. Histograms display the measured fluorescence intensity along the white line in the merged panels.

(D and E) HeLa cells were stimulated with salmon sperm dsDNA by transfection. At 6 h after stimulation, cells were fixed and stained with anti-p-TBK1 and MFN-1 (D) or PSEN-1 antibodies (E). Histograms display the measured fluorescence intensity along the white line in the merged panels.

(F) Colocalization of coefficients of p-TBK1 with IPS-1, STING, MFN-1 or PSEN-1 are shown (mean ± sd, n = 3).

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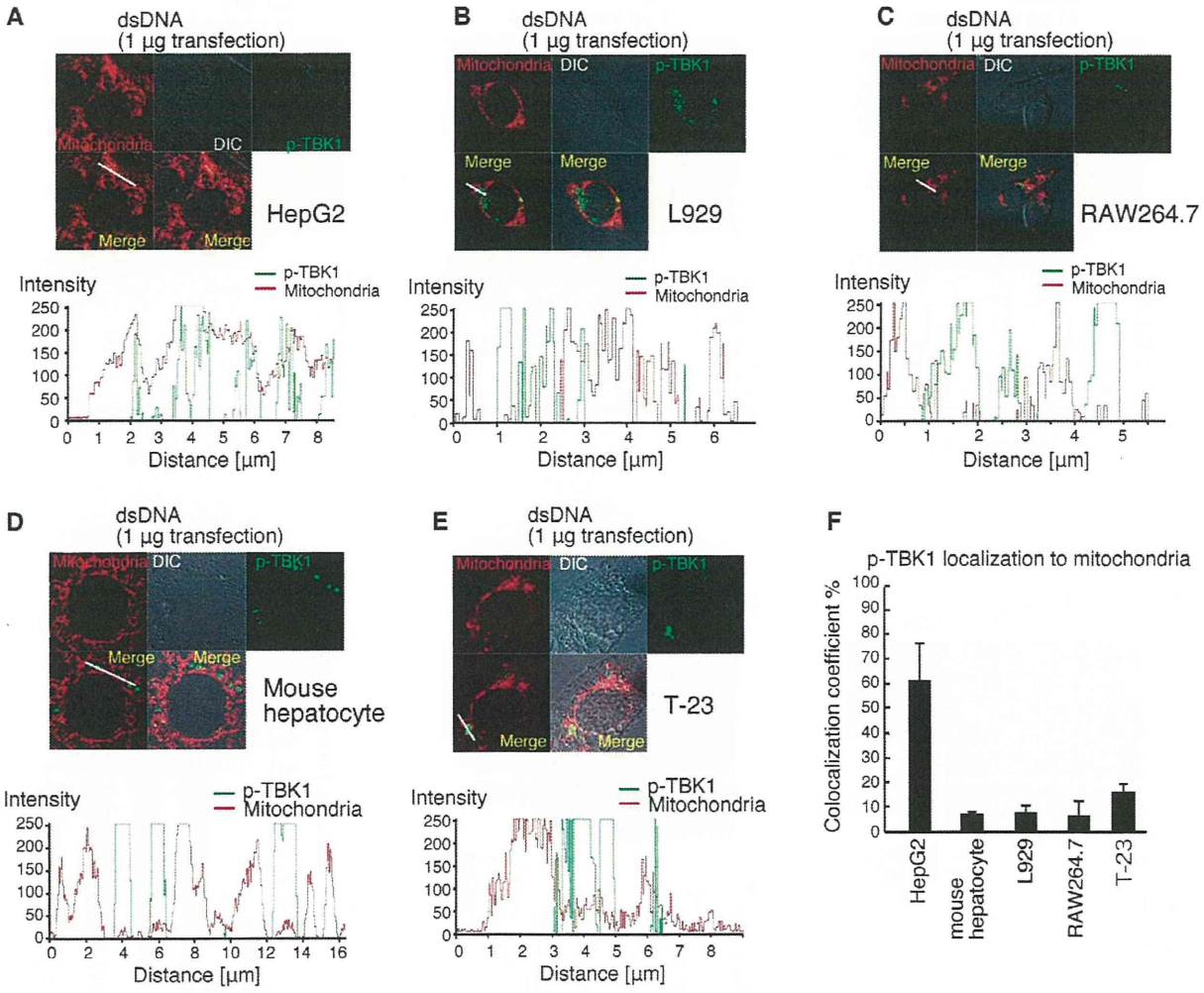

Figure 4. Cell type-specific localization of p-TBK1 in response to cytoplasmic dsDNA. (A-E) HepG2 (A), L929 (B), RAW264.7 (C), mouse hepatocyte (D), and T-23 (E) cells were stimulated with salmon sperm dsDNA by transfection. At 6 h after stimulation, cells were fixed and stained with anti-p-TBK1 antibody and Mitotracker Red. Histograms display the measured fluorescence intensity along the white line in the merged panels.
(F) Colocalization coefficients of p-TBK1 with mitochondria are shown (mean ± sd, n = 3).
doi: 10.1371/journal.pone.0083639.g004

These data suggested that p-TBK1 exhibited cell type-specific localization in response to cytoplasmic DNA.

p-TBK1 mitochondrial localization in response to cytoplasmic viral DNA in human cell lines

Next, we investigated p-TBK1 localization in response to viral DNA. Hepatitis B virus (HBV) is a DNA virus, and its protein HBx suppresses IFN-β mRNA expression in response to dsDNA but not to dsRNA [36], suggesting that DNA sensing pathway is targeted by HBV. When HBV full-length genomic DNA was transfected into HepG2, type I IFN mRNA expression was hardly induced (Figure S2). To avoid the suppression of innate immune response by HBV proteins transcribed from full-length HBV DNA, we used partial fragments of HBV genomic DNA (F1-F4) (Figure 6A). Stimulation with HBV genomic DNA fragments (F1-F4) efficiently induced *IFN-β* mRNA expression (Figure 6A and 6B). As observed with vertebrate DNA, the HBV genomic DNA fragment F1 induced mitochondrial localization of p-TBK1 (Figure 6C and 6D), and most p-TBK1 colocalized with IPS-1 but not STING in HepG2 cells (Figure 6C and 6D). When RAW264.7, the mouse hepatocyte cell line, or T-23 cells were stimulated with the HBV DNA fragment by transfection, p-TBK1 did not exhibit mitochondrial localization (Figure 7A, 7C,

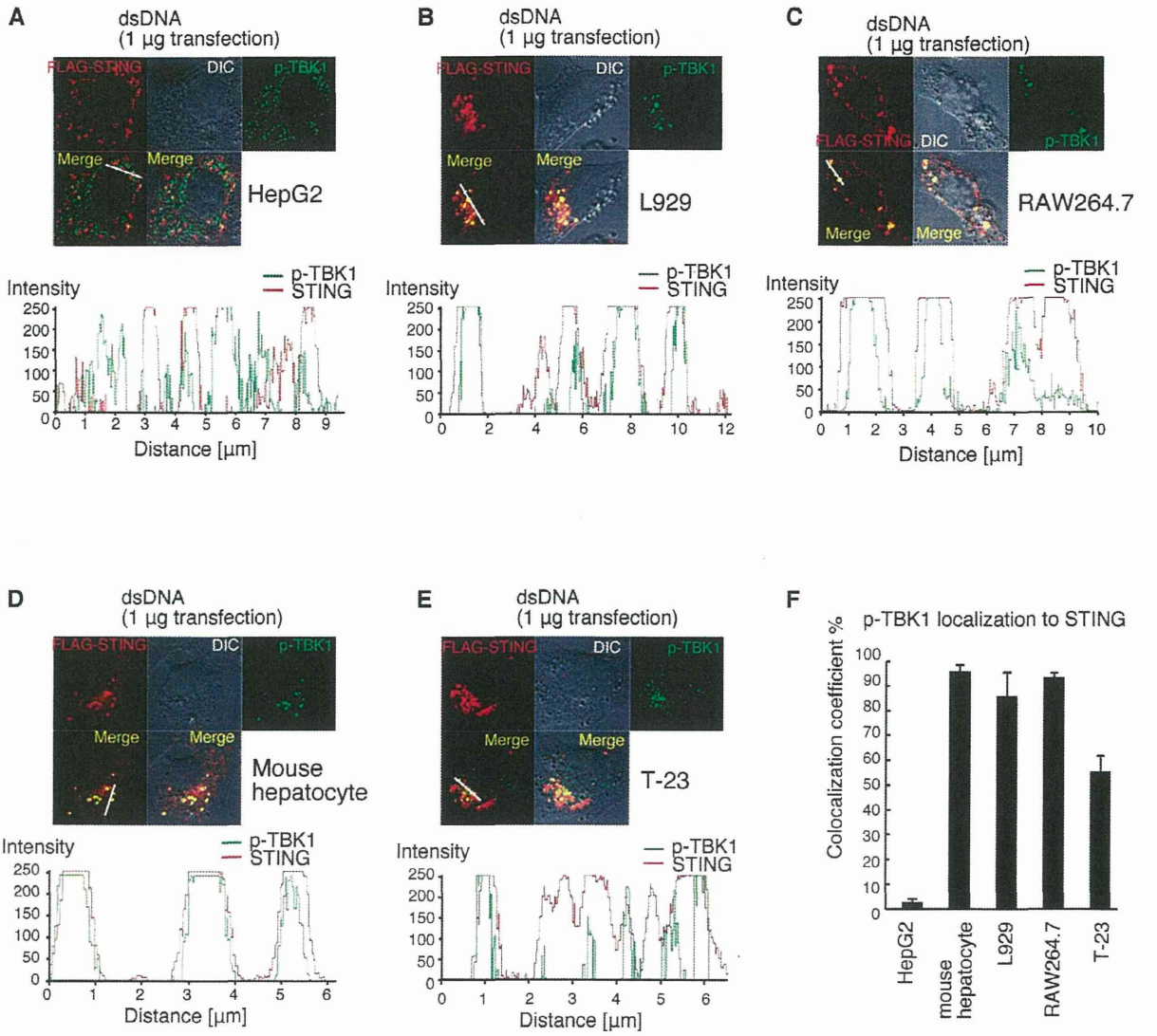

Figure 5. p-TBK1 colocalization with STING in mammalian cells in response to DNA. (A-E) 0.5 μg of FLAG-tagged STING expression vector was transfected into HepG2 (A), L929 (B), RAW264.7 (C), mouse hepatocytes (D) or T-23 (E) cells. At 24 h after transfection, cells were stimulated with salmon sperm dsDNA. At 6 h after transfection, cells were fixed and stained with anti-FLAG and p-TBK1 antibodies. Histograms display the measured fluorescence intensity along the white line in the merged panels. (F) Colocalization coefficients of p-TBK1 with STING are shown (mean ± sd, n = 3).

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and 7E), and more than 70% of p-TBK1 colocalized with STING (Figure 7B, 7D, and 7F).

Next, we investigated p-TBK1 localization after a DNA virus herpes simplex virus type-1 (HSV-1) infection in HeLa, HepG2, the mouse hepatocyte cell line, and T-23 cells. In HeLa and HepG2 cells, p-TBK1 localized on mitochondria, whereas, in the mouse hepatocyte cell line and T-23 cells, most p-TBK1 failed to localize on mitochondria (Figure 8A-8D). The statistic analysis indicated that the colocalization coefficient of p-TBK1 to mitochondria of HeLa or HepG2 cells was higher than that of mouse hepatocyte or T-23 cells (Figure 8E). Taken together, these data indicated that p-TBK1 exhibited mitochondrial localization in response to cytoplasmic viral DNA in HeLa and HepG2 cells, but not in RAW264.7, the mouse hepatocyte cell line, or T-23 cells.

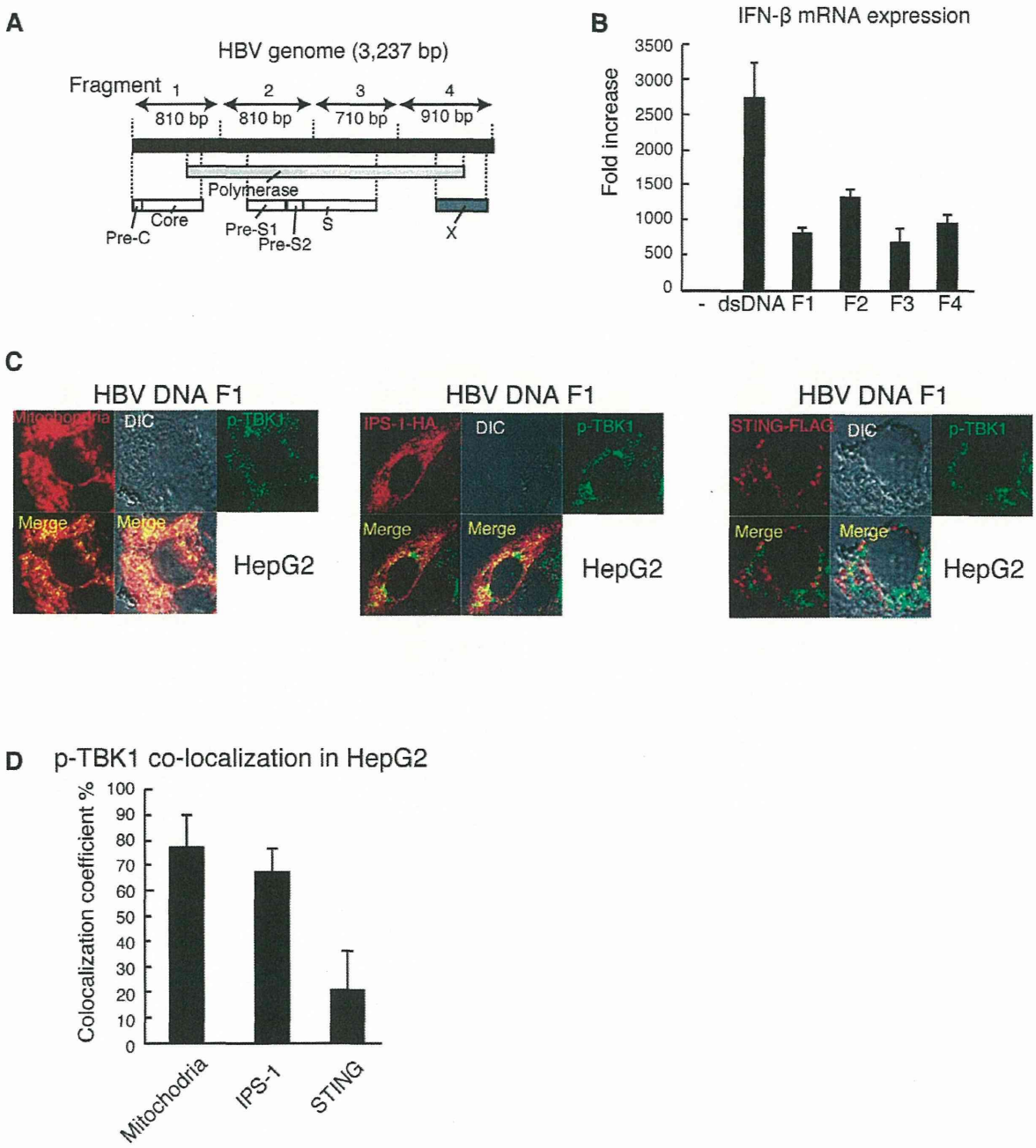

Figure 6. p-TBK1 colocalization with IPS-1 in response to a HBV genomic DNA fragment in HepG2 cells. (A) A schematic diagram of HBV genomic DNA fragments used in panels B-J.

(B) RAW264.7 cells were transfected with mock, salmon sperm dsDNA, or HBV dsDNA fragments F1, F2, F3, or F4. At 6 h after transfection, IFN-β mRNA expression was determined by RT-qPCR.

(C) HepG2 cells were transfected with 1 μg of HBV fragment 1 in 24-well plate. At 6 h after transfection, cells were fixed and stained with anti-p-TBK1 antibody and Mitotracker Red or anti-HA antibody. To observe IPS-1 and STING localization, 0.5 μg of HA-tagged IPS-1 or FLAG-tagged STING expression vector was transfected into HepG2 cells 24 h before stimulation.

(D) Colocalization coefficients of p-TBK1 with mitochondria, IPS-1, or STING (mean ± sd, n = 3).

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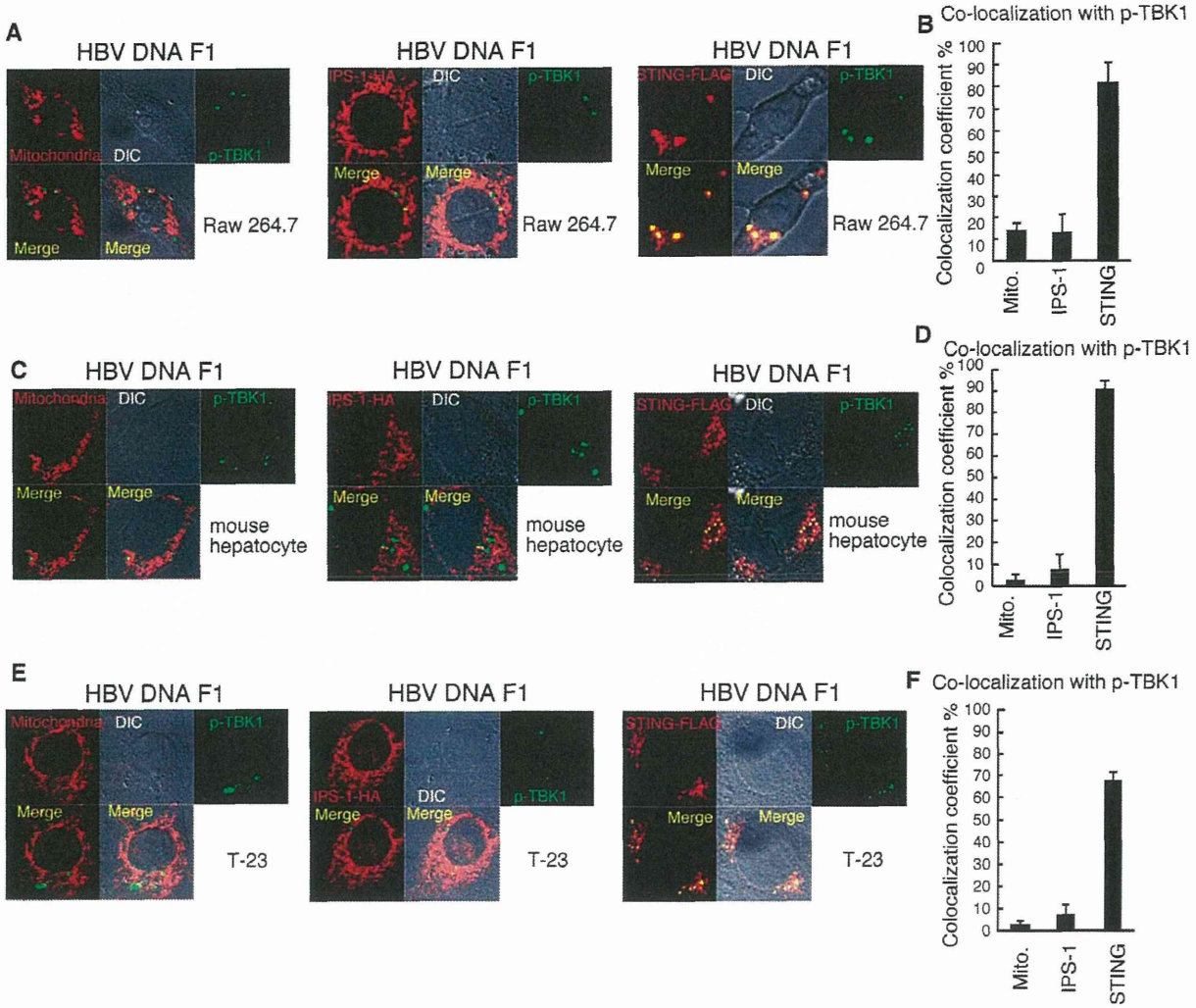

Figure 7. p-TBK1 colocalization with STING in response to a HBV genomic DNA fragment in mammalian cells. RAW264.7 (A and B), mouse hepatocyte (C and D), and T-23 (E and F) cells were transfected with HBV fragment 1. At 6 h after transfection, cells were fixed and stained with anti-p-TBK1 and FLAG antibodies and Mitotracker Red. To observe STING and IPS-1 localization, 0.5 μg of HA-tagged IPS-1 or FLAG-tagged STING expression vectors were transfected into RAW264.7 cells 24 h before stimulation. Colocalization coefficients of p-TBK1 with mitochondria (Mito.), IPS-1, or STING (mean ± sd, n = 3) in RAW264.7 (B), mouse hepatocyte (D), and T-23 (F) cells are shown.

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IPS-1 is essential for TBK1 phosphorylation in HeLa cells in response to cytoplasmic DNA

As most p-TBK1 colocalized with IPS-1 in response to cytoplasmic DNA in HeLa cells, we investigated whether IPS-1 was required for TBK1 phosphorylation in response to cytoplasmic DNA in HeLa cells. We transfected siRNA for negative control or *IPS-1* into HeLa cells. At 48 h after transfection, cells were stimulated with the HBV DNA fragment F1 or salmon sperm dsDNA by transfection. We confirmed that siRNA for *IPS-1* markedly reduced *IPS-1* mRNA levels in both mock and dsDNA stimulated cells (Figure S3). Interestingly, siRNA for *IPS-1* reduced p-TBK1 staining in HeLa cells (Figure 9A). As STING is essential for Type I IFN expression in response to cytoplasmic DNA, we investigated the requirement for STING in TBK1 phosphorylation in response to HBV DNA. Our results showed that siRNA for *STING* reduced STING mRNA levels and abrogated p-TBK1 staining (Figure 9A and Figure S3). Next, we investigated p-TBK1 levels in cell lysates by western blotting and found that knockdown of *IPS-1* reduced p-TBK1 levels induced by DNA stimulation (Figure 9B). siRNA for *STING* also reduced p-TBK1 levels (Figure 9C)

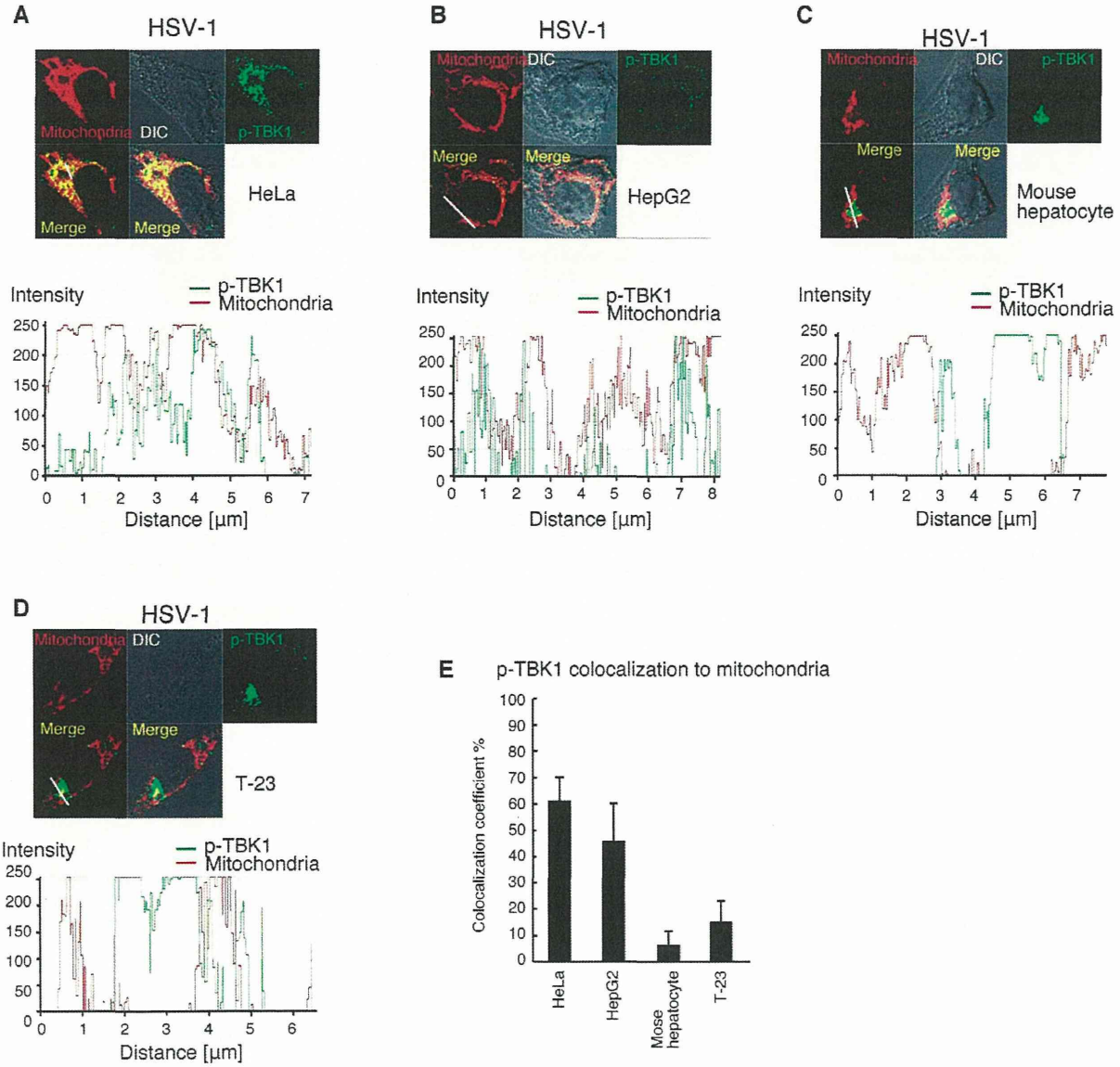

Figure 8. p-TBK1 localization in response to HSV-1 infection. HeLa (A), HepG2 (B), mouse hepatocyte (C), and T-23 (D) cells were infected with HSV-1 for 24 h. Cells were fixed and stained with Mitotracker Red and anti-p-TBK1 antibody. Histograms display the measured fluorescence intensity along the white line in the merged panels. Colocalization coefficients of p-TBK1 to mitochondria are shown (E).

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Next, we investigated whether siRNA for *IPS-1* or *STING* reduces IFN-β mRNA expression in response to DNA stimulation. siRNA for *IPS-1* significantly reduced IFN-β mRNA expression in response to vertebrate DNA and HBV DNA F1 fragment in HeLa cells (Figure 9D), whereas siRNA for *IPS-1* failed to reduce IFN-β mRNA expression in response to vertebrate DNA in L929 cells (Figure S3B). siRNA for *STING* also reduced IFN-β mRNA expression in HeLa cells (Figure 9D). Taken together, these data indicated that IPS-1, as well as STING, are required for TBK1 phosphorylation and efficient IFN-β mRNA expression in response to cytoplasmic DNA in HeLa cells.

Next, we compared dsDNA-induced type I IFN mRNA expressions among human and mouse cells. HeLa cells were less responsive to DNA stimulation compared to THP-1, L929, and RAW264.7 cells (Figure 10A). Therefore, there is a

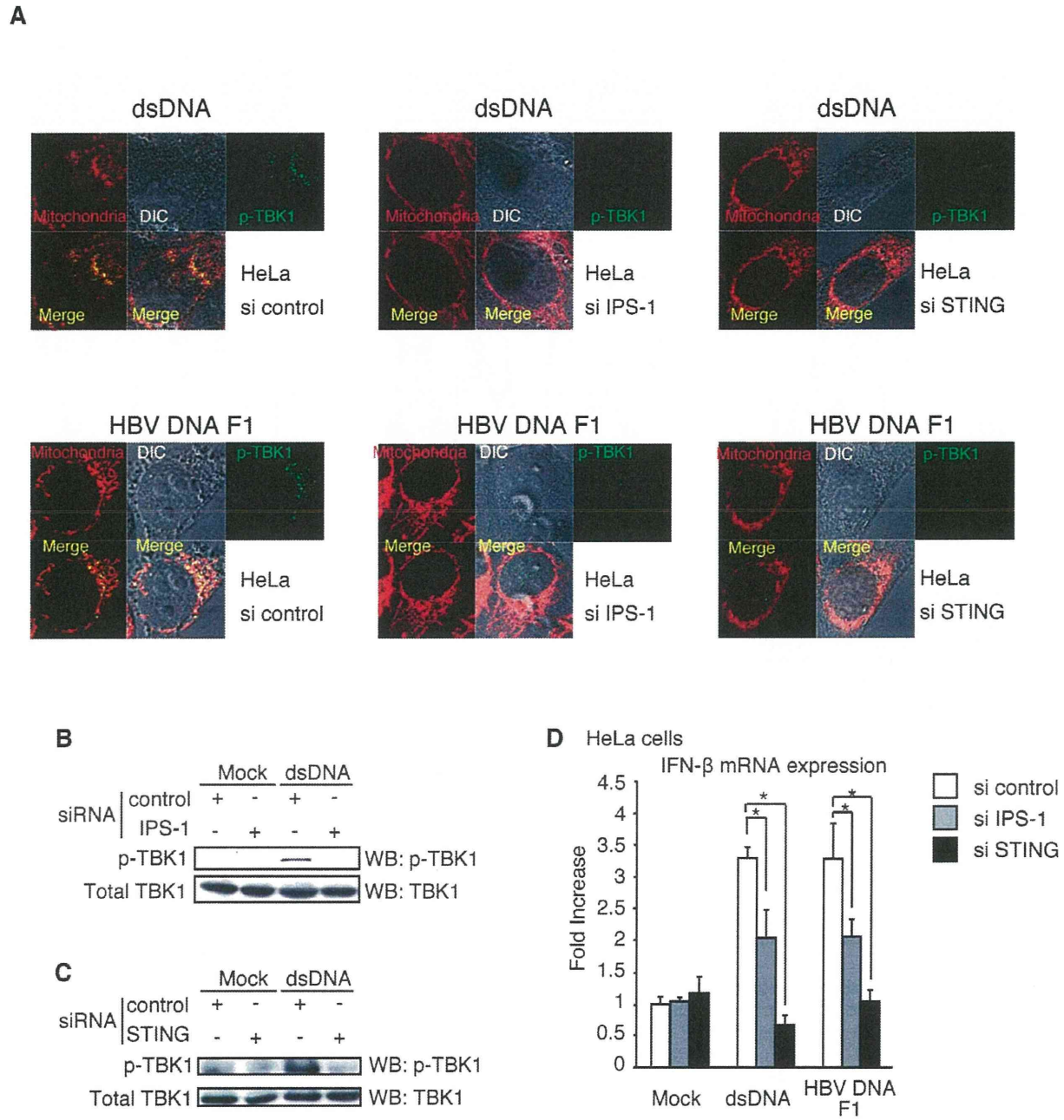

Figure 9. p-TBK1 levels in IPS-1 or STING knockdown cells. (A-C) siRNA for negative control, IPS-1, or STING were transfected into HeLa cells. At 48 h after transfection, cells were stimulated with HBV F1 fragment or salmon sperm DNA for 6 h. Cells were fixed and stained with Mitotracker Red and anti-p-TBK1 antibody (A), or cell lysates were prepared and subjected to SDS-PAGE and western blotting (B and C).

(D) siRNA for IPS-1 or STING were transfected into HeLa cells At 48 h after transfection, cells were stimulated with mock, HBV F1 fragment, or salmon sperm DNA (dsDNA) for 6 h. The IFN-β mRNA expression was determined by RT-qPCR.

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possibility that mitochondrial localization might correlate with a lack of strong response of the cells to the cytoplasmic DNA stimulation. To test this possibility, we investigated p-TBK1 subcellular localization in THP-1, which efficiently expressed IFN-β mRNA in response to DNA stimulation as L929 cells (Figure 10A). p-TBK1 showed mitochondrial localization in response to salmon sperm DNA or HBV F1 DNA fragment in THP-1 cells (Figure 10B). This observation weakened the possibility.

Discussion

Autophosphorylation of TBK1 is essential for Type I IFN expression [28]. Here, we demonstrated that p-TBK1 is localized on mitochondria in response to cytoplasmic DNA in HeLa and HepG2 cells. p-TBK1 induced by DNA stimulation in HeLa and HepG2 cells co-localized with IPS-1 and MFN-1. Moreover, knockdown of *IPS-1* reduced p-TBK1 levels in response to viral DNA in HeLa cells. These data indicate that IPS-1 plays a crucial role in the phosphorylation of TBK1 in response to cytoplasmic DNA in the human cells. In contrast, p-TBK1 did not localize on mitochondria in the mouse or tree shrew cells that we tested. This is consistent with a previous knockout mouse study that showed that IPS-1 is dispensable for the response to cytoplasmic DNA [33]. Thus, there appears to be species-specific mechanisms of Type I IFN production in response to cytoplasmic DNA. However, we do not exclude the possibility that some of human primary or immortalized cells do not exhibit IPS-1 mitochondrial localization in response to cytoplasmic viral DNA. Taniguchi and colleagues firstly reported cell type-specific roles of a DNA sensor, DAI, in a cytoplasmic DNA sensing pathway [32,37]. Later, other groups reported several other DNA sensors using various types of cells [38]. Recently Chen and colleagues showed that cGAS is essential for type I IFN production in response to poly(dA:dT) in plasmacytoid DC but not in lung fibroblasts [39]. Their findings suggested that RNA polymerase III – RIG-I pathway plays a major role in sensing DNA in lung fibroblasts [24]. Thus, these observations indicate that there are several cell type-specific DNA sensing pathways. Our observations of cell type-specific p-TBK1 localization in response to cytoplasmic DNA also support this model.

Gale and colleagues previously reported that a major site of IPS-1 signaling is MAMs [8]. TBK1 autophosphorylation in response to HBV DNA required both IPS-1 and STING in human cells that we tested. Considering that IPS-1 and STING localize on the mitochondria and ER, respectively, it is expected that TBK1 autophosphorylation occurs within MAMs where the ER associates with mitochondria in response to cytoplasmic viral DNA in HeLa and HepG2 cells. Indeed, a fraction of cellular p-TBK1 was localized on MAMs. It is likely that TBK1 moved to mitochondria after autophosphorylation in human cells. In contrast, mouse and tree shrew TBK1 appears to be phosphorylated on ER where STING localizes. The biological significance of the mitochondrial or ER localization of p-TBK1 is unclear. A previous report demonstrated that peroxisomal IPS-1 triggers rapid expression of ISGs, whereas mitochondrial IPS-1 triggers delayed ISG and IFN expression

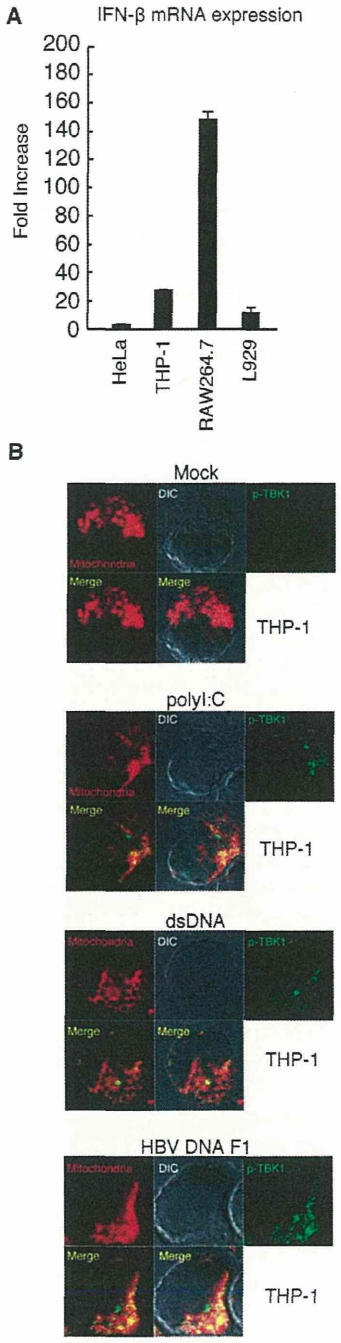

Figure 10. Subcellular localization of p-TBK1 in response to polyI:C or DNA in THP-1 cells. (A) HeLa, THP-1, Raw264.7, and L929 cells were stimulate with salmon sperm DNA for 6 h by transfection. The fold increase of IFN-β mRNA expression in response to DNA was determined by RT-qPCR. (B) THP-1 cells were stimulated with mock, polyI:C, salmon sperm DNA (dsDNA), or HBV DNA F1 for 3 h. Cells were fixed and stained with Mitotracker Red and anti-p-TBK1 antibody.

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[7]. Thus, as observed with IPS-1, differential p-TBK1 placement may allow the cell to diversify signaling pathways.

When human STING or IPS-1 were transfected into mouse cells, p-TBK1 did not localize on the mitochondria in response to cytoplasmic DNA. Thus, the differences in the STING and IPS-1 protein sequences between humans and mice are not a cause of the cell type-specific localization of p-TBK1. It is possible that the difference in TBK1 protein sequence between humans and mice determines the cell type specific localization of p-TBK1. Another possibility is that an unknown factor of human or mouse associate with TBK1 or p-TBK1. Our current knowledge cannot explain the cell type-specific localization of p-TBK1 in response to cytoplasmic DNA stimulation. Further study is required to reveal the precise mechanisms used by cytoplasmic DNA sensing pathways.

Materials and Methods

Cells, viruses, and reagents

A tree shrew (*Tupaia belangeri*) fibroblast cell line, T-23 (clone 8) cells were obtained from JCRB. T-23 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium low glucose medium (D-MEM) with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen). HeLa cells were cultured in minimum Eagle's medium with 2 mM L-glutamine and 10% heat-inactivated FCS. Protocols for the isolation and culture conditions of mouse hepatocytes have been described previously [34]. L929 and RAW264.7 cells were cultured in RPMI1640 with 10% of FCS. Salmon sperm DNA and polyI:C were purchased from Invitrogen or GE Healthcare, respectively. HSV-1 K strain was amplified using Vero cells. To determine viral titers, we performed plaque assay using Vero cells. Vero cells were cultured in D-MEM with 10% of FCS. THP-1 cells were cultured in RPMI1640 with 10 % FCS and 0.1 % 2-mercaptoethanol.

Confocal Microscopy

Phospho-TBK1/NAK (Ser 172) (D52C2) XP rabbit mAb were purchased from Cell Signaling. Anti-Presenilin 1 Ab [APS11], anti-NAK (TBK1) Ab (EP611Y), anti-mitofusin 1 Ab, and anti-G3BP Ab were purchased from Abcam. Anti-FLAG antibody was purchased from Sigma-Aldrich. Anti-HA Ab [HA1.1] was purchased from COVANCE. Mitotracker Red was purchased from Life Technologies. Cells were fixed with 3% of formaldehyde in 1x PBS for 30 min, and permeabilized with 0.2% Triton X-100 in 1 x PBS for 15 min. In case of PSEN-1 staining, fixed cells were permeabilized with 0.5% of saponin in 1x PBS with 1 % BSA for 30 min. For blocking, 1% BSA in PBS was used for 30 min. The cells were labeled with the indicated primary antibody for 60 min at room temperature. After washing four times with 1% BSA in PBS, cells were incubated with an Alexa-conjugated secondary antibody and 1% BSA in PBS for 30 min at room temperature, and then were washed four times with 1% BSA in PBS. Samples were mounted on glass slides using Prolong Gold (Invitrogen). Cells were visualized at a magnification of ×63 with an LSM510 META microscope (Zeiss). Colocalization of coefficients and intensity histograms were determined using LSM510 ZEISS LSM Image examiner software.

Plasmids

RIG-I CARDs expression vector (dRIG-I) has been described previously [40]. Human *IPS-1* cDNA encoding the full-length ORF was cloned into a pEF-BOS multi-cloning site, and an HA sequence was inserted just before the STOP codon. Human *STING* cDNA that encoded a full-length ORF was cloned into a pEF-BOS multi-cloning site, and a FLAG-tag sequence was inserted just before the STOP codon. The plasmids were sequenced to confirm that there were no PCR errors.

HBV DNA preparation and quantitative PCR

HBV DNA fragments were obtained by PCR using HBV DNA as a template. A plasmid carrying HBV full-length genomic DNA were kindly gifted from Chayama K. Primer sequences were as follows: F1 forward, TGC AAC TTT TTC ACC TCT GC; F1 reverse, TCT CCT TTT TTC ATT AAC TG; F2 forward, TTA AAA TTA ATT ATG CCT GC; F2 reverse, AAC AAG AAA AAC CCC GCC TG; F3 forward, GAC AAG AAT CCT CAC AAT AC; F3 reverse, TGT ACA ATA TGT TCT TGT GG; F4 forward, AAA AAT CAA GCA ATG TTT TC; and F4 reverse, ATT AGG CAG AGG TGA AAA AG. Amplified DNA fragments were purified using a Gel Extraction kit (Qiagen). For quantitative PCR (qPCR), total RNA was extracted using TRIZOL reagent (Invitrogen), after which 0.1-1 μg of RNA was reverse-transcribed using a high capacity cDNA transcription kit with an RNase inhibitor kit (Applied Biosystems) according to the manufacturer's instructions. qPCR was performed using a Step 1 real time PCR system (Applied Biosystems). The expression of cytokines mRNA was normalized to that of *GAPDH* or *β-actin* mRNA, and the fold-increase was determined by dividing the expressions in each sample by that of the wild-type at 0 h. The primers used for qPCR were described previously [40,41].

Supporting Information

Figure S1. HeLa cells were transfected with HA-tagged IPS-1 (A) or FLAG-tagged STING (B). At 24 h after transfection, cells were mock-stimulated for 6 h, and then fixed and stained with anti-p-TBK1 and HA or FLAG Abs.
(TIF)

Figure S2. HepG2 cells were transfected with a vector carrying 1.4 x HBV genomic DNA. Total RNA was extracted at indicated hours. IFN-β mRNA expression was determined by RT-qPCR.
(TIF)

Figure S3. siRNAs for control, IPS-1, or STING were transfected into HeLa and L929 cells. 48 h after transfection, cells were stimulated with or without dsDNA for 6 h. Total RNA was extracted with TRIZOL, and RT-qPCR was performed. IPS-1 and STING mRNA expressions were normalized with β-actin mRNA expression. Relative ratio was calculated by