The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING–IFN-β pathway

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Evasion of host immunity is a hallmark of cancer; however, mechanisms linking oncogenic mutations and immune escape are incompletely understood. Through loss-of-function screening of 1,001 tumor suppressor genes, we identified death-associated protein kinase 3 (DAPK3) as a previously unrecognized driver of anti-tumor immunity through the stimulator of interferon genes (STING) pathway of cytosolic DNA sensing. Loss of DAPK3 expression or kinase activity impaired STING activation and interferon (IFN)-β-stimulated gene induction. DAPK3 deficiency in IFN-β-producing tumors drove rapid growth and reduced infiltration of CD103+CD8α+ dendritic cells and cytotoxic lymphocytes, attenuating the response to cancer chemo-immunotherapy. Mechanistically, DAPK3 coordinated post-translational modification of STING. In unstimulated cells, DAPK3 inhibited STING K48-linked poly-ubiquitination and proteasome-mediated degradation. After cGAMP stimulation, DAPK3 was required for STING K63-linked poly-ubiquitination and STING-TANK-binding kinase 1 interaction. Comprehensive phospho-proteomics uncovered a DAPK3-specific phospho-site on the E3 ligase LMO7, critical for LMO7–STING interaction and STING K63-linked poly-ubiquitination. Thus, DAPK3 is an essential kinase for STING activation that drives tumor-intrinsic innate immunity and tumor immune surveillance.

To shed insights into tumor-intrinsic innate immunity, we performed genetic loss-of-function screening of 1,001 tumor suppressor genes in primary human cells, identifying DAPK3 as an essential regulatory kinase in the STING–IFN-β pathway. Tumor-specific loss of DAPK3 expression or kinase activity accelerated the natural growth of IFN-β-producing tumors in immunocompetent and STING-deficient hosts, and increased tumor resistance to STING agonists and STING-activating chemo-immunotherapies, attenuating the efficacy of combination therapy with anti-PD-1. At the molecular level, DAPK3 coordinated STING ubiquitination. Loss of DAPK3 expression, but not its kinase activity, enhanced STING K48-linked poly-ubiquitination and diminished steady-state STING protein levels. DAPK3 deficiency also diminished K63-linked STING poly-ubiquitination following cGAMP stimulation, due to hypo-phosphorylation of the E3 ligase LMO7, which was directly phosphorylated by DAPK3 on S663. Studies establish DAPK3 as an essential activating kinase in the core STING signaling cascade, and a driver of tumor-intrinsic innate immunity and tumor immune surveillance. Thus, DAPK3 loss-of-function observed in several human tumor types drives evasion of host immunity and diminished efficacy of cancer immunotherapy.

Results

DAPK3 is a positive regulator of STING signaling. To examine tumor-intrinsic regulation of STING, we performed genetic loss-of-function screening of 1,001 human tumor suppressor genes (Supplementary Table 1 and Extended Data Fig. 1a). Inducible nuclear IRF3 translocation was quantified in RNA interference–treated cells, and it was found that DAPK3 loss of function increased IRF3 nuclear translocation. These findings were confirmed in primary human cells, where DAPK3 loss of function increased IRF3 nuclear translocation. Our studies suggest that DAPK3 loss of function promotes the activation of the STING pathway, leading to the induction of IFN-β and other cytokines. These results highlight the importance of DAPK3 in the regulation of STING signaling and its potential role in the development of cancer.

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Fig. 1 | DAPK3 regulates DNA-stimulated STING signaling. a, RNA interference screen of 1,001 tumor suppressor genes in HUVECs, represented as ranked mean Z-score for poly (dA:dT)-induced IRF3 nuclear translocation (0.5 μg ml⁻¹) for 3 h. b, c, IRF3 nuclear translocation in HUVECs (b) and L929-mRuby-hIRF3 (c) stimulated with poly (dA:dT) (0.5 μg ml⁻¹), VACV70 (2 μg ml⁻¹) or 2′,3′-cGAMP (10 μg ml⁻¹) or infected with hCMV (MOI=5) for 3 h. d, f, RT-qPCR of IFNB1 (d), CXCL10 (e) and CCL5 (f) in HUVECs transfected with indicated siRNA and stimulated with poly (dA:dT) (0.5 μg ml⁻¹), VACV70 (2 μg ml⁻¹) or 2′,3′-cGAMP (10 μg ml⁻¹) for 4 h. g–i, RT-qPCR of Ifnb1 (g), Cxcl10 (h) and Mx2 (i) in L929-mRuby-hIRF3 transfected with indicated siRNA and stimulated with poly (dA:dT) (0.5 μg ml⁻¹), VACV70 (2 μg ml⁻¹) or 2′,3′-cGAMP (10 μg ml⁻¹) for 4 h. j–l, RT-qPCR of IFNB1 (j), CXCL10 (k) and CCL5 (l) in THP1-Blue ISG cells transduced with indicated shRNA and stimulated with poly (dA:dT) (0.5 μg ml⁻¹), VACV70 (2 μg ml⁻¹) or 2′,3′-cGAMP (10 μg ml⁻¹) for 4 h. m, n, RT-qPCR of Ifnb1 in MCA205 (m) and B16F10 (n) cells transduced with the indicated shRNA and stimulated with 2′,3′-cGAMP (10 μg ml⁻¹), 3′,5′-cGAMP (10 μg ml⁻¹) or DMXAA (50 μg ml⁻¹) for 4 h. mRNA levels were normalized to values of ACTB (human) and Rn18s or Actb (mouse), and percentage expression was calculated from stimulated control values. Data in b–n represent the mean of three independent experiments. Values represent mean ± s.d. * P < 0.05, ** P < 0.01 and *** P < 0.001. Statistical comparisons were conducted using two-tailed t-test (b–n). TSG, tumor suppressor gene.
human umbilical vein endothelial cells (HUVECs) by in situ immunofluorescence after 3 h of stimulation with poly (dA:dT) DNA (Extended Data Fig. 1b), which requires expression of cGAS, STING, and TBK1 (ref. 19). From these studies DAPK3 emerged as a positive regulator of DNA-stimulated IRF3 nuclear translocation (Fig. 1a). The DAPK family of serine/threonine kinases includes DAPK1, DAPK2 and DAPK3, which regulate cellular apoptosis and autophagy20. Quantitative PCR with reverse transcription (RT–qPCR) indicated broad expression of DAPK1/Dapk1 and DAPK3/Dapk3 in human and mouse cells (Extended Data Fig. 1c,d), while indicated broad expression of DAPK3/Dapk3 and IFNB1/Ifnb1, induction of IFN-β signaling by MCA205 T cells and M2 macrophages (Extended Data Fig. 1e), suggesting a pleiotropic function of DAPK3 in a subpopulation of hematopoietic cells21.

To validate screen results, HUVECs were transfected with short interfering RNAs (siRNAs) targeting DAPK3 or STING1, and IRF3 activation further assessed. DAPK3 depletion diminished IRF3 activation in response to poly (dA:dT) DNA and VACV70 DNA, the natural STING agonist 2′,3′-cGAMP or infection with human cyto-megalovirus (hCMV), a DNA-encoding virus that engages cGAS–STING signaling22 (Fig. 1b). In mouse L929-mRuby-hIRF3 reporter cells (hereafter called L929) (Extended Data Fig. 1e), depletion of DAPK3 using two sequence-independent lentiviral short hairpin RNAs (shRNAs) impaired IRF3 nuclear translocation induced by DNA and 2′,3′-cGAMP (Fig. 1c). Consistent with these findings, induction of IFNB1/Ifnb1 and Interferon-stimulated genes (ISGs) CXCL10/Cxcl10, CCL5/Ccl5, MX2/Mx2 and IL6/Il6 was significantly impaired in HUVECs, L929 and THP1-Blue ISG reporter cells (hereafter called THP1) after DAPK3 depletion (Fig. 1d–l and Extended Data Fig. 1j,k), with effects comparable to STING. Similar results for Ifnb1 were obtained in mouse tumor cell lines MCA205 and B16F10 (Fig. 1m,n). DAPK3 was described as a negative regulator of cytosolic dsRNA sensing through RIG-I (ref. 19). In HUVECs and mouse bone marrow-derived macrophages (BMDMs), which express both DAPK1/Dapk1 and DAPK3/Dapk3, IFNB1/Ifnb1 was increased upon DAPK3 depletion in response to poly (dA:dT) DNA (Extended Data Fig. 1f–i), indicating that DAPK3 and DAPK1 exert distinct effects on IFN-β induction by DNA. In DAPK3-depleted L929 and THP1, IFNB1/Ifnb1 induced by cytosolic double-stranded RNA ligands was unaffected (Extended Data Fig. 1l,m,p). However, DAPK3 positively regulated TLR3 and TLR4 signaling in THP1 (Extended Data Fig. 1n,o), suggesting a pleiotropic role in innate immune signaling. Results identify DAPK3 as an activating kinase in the STING–IRF3–IFN-β pathway in human and mouse cells.

**Tumor-expressed DAPK3 shapes immune surveillance.** Dapk3 whole-body knockout in mice is embryonically lethal19 and we were consistently unable to maintain knockout lines generated using CRISPR–Cas9, suggesting an essential role in cellular growth. However, loss-of-function DAPK3 mutations are frequently observed in human tumors22, and lower DAPK3 expression was associated with significantly shorter survival times in patients with pancreatic carcinoma, esophageal carcinoma and uterine corpus endometrial carcinoma (Extended Data Fig. 2).

**Growth phenotypes were analyzed in MCA205 and B16F10 depleted of DAPK3 using lentiviral shRNAs.** In both lines, in vitro proliferation was impaired upon loss of DAPK3 but not STING (Extended Data Fig. 3a,b), likely due to specific effects upon cytokinesis23. Despite inhibitory effects in vitro, growth of MCA205 tumors depleted of DAPK3 was significantly accelerated in vivo (Fig. 2a), while B16F10 tumors were unaffected (Fig. 2b). In MCA205 tumors depleted of DAPK3 or STING, significant reduction in numbers of anti-tumor natural killer cells, CD8+ T cells and CD103+CD8α+ dendritic cells was accompanied by significant increase in numbers of pro-tumor regulatory T cells and M2 macrophages (Fig. 2c and Extended Data Fig. 4). To examine the contribution of host IFN-β signaling to MCA205 tumor growth, type I IFN receptor (Ifnar1)-knockout mice were examined. Compared with wild-type (WT) mice, growth advantage of DAPK3-depleted MCA205 tumors was eliminated in the absence of host IFNAR signaling (Fig. 2d), suggesting that tumor-derived IFN-β activates paracrine signaling in host immune cells to drive spontaneous immune rejection of MCA205 tumors. Accordingly, numbers of tumor-infiltrating CD8+ T cells and CD103+CD8α+ dendritic cells were significantly reduced in Ifnar1-knockout mice, and differences between control and DAPK3- or STING-depleted MCA205 tumors attained (Extended Data Fig. 3c).

MCA205 cells derive from fibrosarcomas in mice treated with the DNA alkylating agent methylcholanthrene. Due to dependence of natural MCA205 tumor growth upon intrinsic DAPK3–STING–IFN-β signaling, which was not observed in B16F10, we reasoned that genomic instability might drive spontaneous activation of this axis. Confocal microscopy of MCA205-cGAS-Clover reporter cells showed constitutive colocalization of cGAS with yH2AX, a marker of DNA damage-induced micronucleus formation24, in unstimulated cells (Extended Data Fig. 3d). Accordingly, baseline Ifnb1 levels in unstimulated MCA205 were sensitive to DAPK3 or STING depletion, in contrast to B16F10 (Extended Data Fig. 3e). Further, ectopic expression of a kinase-dead DAPK3 D161A point mutant initially identified in human ovarian tumors25,26 diminished baseline and stimulated ifnb1 expression (Extended Data Fig. 3f). To assess the functional impact of oncogenic DAPK3 mutations on tumor immunology, DAPK3-depleted MCA205 cells were transduced with lentiviruses encoding human DAPK3(WT) or kinase-dead DAPK3(D161A) before engraftment into mice. Expression of DAPK3(D161A) significantly accelerated in vivo tumor growth compared with DAPK3(WT), which depended upon host IFNAR1 signaling (Fig. 2e). Intracellular staining for double-positive phospho-TBK1 and phospho-IRF3 MCA205-GFP tumor cells confirmed that tumor-intrinsic IRF3 activation was attenuated by DAPK3 depletion (Fig. 2f), while tumor-infiltrating dendritic cells were comparable to controls. Results demonstrate that functional DAPK3 expression is necessary for STING-driven immune surveillance in IFN-β-producing tumors such as MCA205.

STING depletion did not affect natural growth of B16F10 tumors in vivo (Fig. 2b), consistent with relatively low levels of basal Ifnb1 produced in culture (Extended Data Fig. 3e). Treatments that boost anti-tumor immunity by activating tumor-intrinsic cGAS–STING signaling have been reported. Cyclic di-nucleotide (CDN) STING agonists enhance T cell-dependent anti-tumor immunity by targeting innate cells in the tumor microenvironment27, and are currently being explored in clinical cancer trials in combination with immune checkpoint blockade (ID: NCT03010176, NCT03937141). To activate STING, bacterial CDN 3′,5′-cGAMP was injected into B16F10 tumors. While control tumors showed growth delay, anti-tumor effects of CDN were partially blunted in tumors depleted of DAPK3 or STING (Fig. 3a), suggesting that CDN targets tumor cells and host immune cells in the tumor microenvironment28. Accordingly, when transplanted in STING−/− mice, DAPK3-depleted tumors lost all CDN responsiveness (Fig. 3a).

Recent studies indicate that chemo-immunotherapeutic agents activate cGAS–STING signaling in tumors. The Topoisomerase II inhibitor teniposide and the anti-microtubule agent paclitaxel drive mitotic arrest and micronucleus formation and directly activate cGAS11,21–23. Confocal analysis of B16F10-cGAS-Clover treated with either teniposide or paclitaxel confirmed colocalization of cGAS and yH2AX (Extended Data Fig. 2g), and depletion of DAPK3 or STING impaired Ifnb1 and Cxcl10 or Il6 induction by teniposide or paclitaxel, respectively (Fig. 3b,c). Teniposide-induced phosphorylation of IRF3 (Fig. 3d), paclitaxel-induced phosphorylation of p65 (Fig. 3e) and STAT1 phosphorylation at Y701 (refs. 11,23) (Fig. 3d,e) were significantly attenuated by depletion of DAPK3 or STING, while inducible yH2AX levels and cell death were comparable
Fig. 2 | Tumor-expressed DAPK3 shapes immune surveillance. **a**, Tumor volume of shRNA-transduced MCA205 (a) and B16F10 (b) subcutaneously transplanted into C57BL/6J WT mice (n = 8 per group). **c**, Flow cytometry of tumor-infiltrating leukocytes in MCA205 tumor suspensions isolated from WT mice on day 6 (n = 7 for shControl, n = 8 for shDapk3#1 and shSting1#1). **d**, Tumor volume of shRNA-transduced MCA205 subcutaneously transplanted into WT and Ifnar1-KO mice (n = 8 per group). **e**, Tumor volume of shDapk3#1-transduced MCA205 rescued with lentiviral DAPK3 (WT) or DAPK3 (D161A) before subcutaneous transplantation into WT mice and Ifnar1-KO mice (n = 8 per group). **f**, Flow cytometry of intracellular pTBK1 and pIRF3 in MCA205-GFP tumor cells (CD45−CD11c−). Values represent percentage of each total cell population. Data in **a–f** are representative of three independent experiments. Values represent mean ± s.d. *P < 0.05, **P < 0.01 and ***P < 0.001. Statistical comparisons were conducted using two-tailed t-test (**a–f**). DC, dendritic cell; KO, knockout; NK, natural killer; NS, not significant; Treg, regulatory T cell.

(Extended Data Fig. 3h). In B16F10 tumors, single administration of teniposide or paclitaxel showed significant anti-tumor effects in a host IFNAR-dependent manner (Extended Data Fig. 3i), which was attenuated by tumor-intrinsic loss of DAPK3 or STING (Fig. 3j,h,i). Single administration of anti-PD-1 demonstrated comparable anti-tumor effects in DAPK3- or STING-depleted tumors (Fig. 3g); however, the synergistic effects of anti-PD-1 and teniposide or paclitaxel were abolished (Fig. 3j,k). Tumor-infiltrating CD8+T cells and CD103+CD8α+ dendritic cell numbers increased by teniposide or paclitaxel treatment were significantly blunted by loss of DAPK3 or STING (Fig. 3i). Chemo-immunotherapeutic agents increased percentage of double-positive phospho-TBK1 and phospho-IRF3 in B16F10-GFP tumor cells, which was reduced by DAPK3 or STING depletion (Fig. 3m). Collectively, data show that functional DAPK3 expression is critical for tumor-intrinsic cGAS–STING-driven responses during natural and chemo-immunotherapy-induced immune rejection of tumors.

DAPK3 inhibits STING K48-linked poly-ubiquitination. We next examined molecular mechanisms by which DAPK3 regulates STING. Immunoblotting demonstrated that DAPK3 depletion in HUVEC and MCA205, but not in BMDM or B16F10, reduced steady-state STING protein levels (Extended Data Figs. 1g,i and 3j). Loss of DAPK3 decreased STING protein levels and IRF3 nuclear translocation in cells treated with DAPK3-specific shRNAs (Extended Data Fig. 5b,c) or siRNAs (Fig. 1c and Extended Data Fig. 5e). DAPK3 depletion also impaired...
DNA-induced NFκB p65 nuclear translocation (Extended Data Fig. 5d). Consistent with loss of STING protein, DAPK3 depletion in L929 inhibited VACV70-induced TBK1 and IRF3 phosphorylation (Extended Data Fig. 5f). Results indicate that DAPK3 maintains steady-state STING protein levels in some cell types, including HUVEC, MCA205 and L929.
**Fig. 4** | DAPK3 inhibits STING K48-linked poly-ubiquitination. 

**a.** Immunoblot of L929-mRuby-hIRF3 cells transduced with the indicated shRNA. Data from two gels are presented. 
**b.** Immunoblot of L929-mRuby-hIRF3 cells transduced with indicated shRNA treated with MG132 (20 μM), lactacystin (5 μM) or vehicle for 4 h. 
**c.** Immunoprecipitation and immunoblot of L929-mRuby-hIRF3 cells transduced with indicated shRNA treated with MG132 (20 μM) for 4 h (upper), and immunoblot of whole-cell lysates (WCLs) (lower). Endogenous K48-linked poly-ubiquitination chains were immunoprecipitated using K48-TUBE-Flag. 
**d.** Immunoprecipitation and immunoblot of L929-mRuby-hIRF3 with or without poly (dA:dT) stimulation for 2 h and 4 h (upper), and immunoblot of WCL (lower). 
**e.** Immunoblot of L929-mRuby-hIRF3 cells transduced with the indicated shrNA. Data from two gels are presented. 
**f.** Immunoblot of L929-mRuby-hIRF3 transduced with indicated shrNA treated with MG132 (20 μM) or vehicle for 4 h. 

**g.** Immunoblot of L929-mRuby-hIRF3 cells transduced with indicated shrNA. Data from two gels are presented. 

**h.** Immunoblot of THP1-Blue ISG cells expressing V5-tagged DAPK3(WT) or DAPK3(D161A) stimulated with poly (dA:dT) (0.5 μg/ml−1) or VACV70 (2 μg/ml−1) for 3 h. 

Treatment of DAPK3-depleted L929 with proteasome inhibitors MG132 and lactacystin restored STING protein levels (Fig. 4b), suggesting that proteasome-mediated degradation of STING is enhanced in DAPK3-depleted cells. We examined K48-linked poly-ubiquitination, which controls steady-state STING degradation. K48-linked poly-ubiquitinated proteins immunoprecipitated from lysates prepared from unstimulated DAPK3-depleted L929 were enriched for K48-ubiquitinated STING (Fig. 4c). Accordingly, endogenous DAPK3 interacted with endogenous STING in unstimulated L929, which was enhanced by DNA stimulation (Fig. 4d). To examine kinase activity, DAPK3-depleted L929 cells were treated with lentiviruses encoding human DAPK3(WT), kinase-dead DAPK3(D161A) or kinase-deficient DAPK3(T180A) (ref. 25). Expression of kinase mutants restored STING protein levels to that of WT in L929 (Fig. 4e) and MCA205 (Extended Data Fig. 3f), indicating that DAPK3 kinase activity is dispensable for maintaining steady-state STING protein levels. However, DNA-induced IRF3 nuclear translocation was still impaired in cells expressing kinase mutants (Fig. 4f). Concordant with this, although STING protein levels in D161A-expressing HUVEC and THP1 were comparable to WT (Extended Data Fig. 5a), dominant negative effects were observed on IRF3 nuclear translocation (Fig. 4g) and IFNB1 induction after poly (dA:dT) (0.5 μg/ml−1) stimulation (Fig. 4h). Data a–d and g are representative of or in f and h are the mean of three independent experiments. Values represent mean ± s.d. **P < 0.01 and ***P < 0.001. Statistical comparisons were conducted using two-tailed t-test (f, h). IP: immunoprecipitation; MOI, multiplicity of infection.
Fig. 5 | DAPK3 promotes STING K63-linked poly-ubiquitination.  

**a.** Immunoblot of THP1-Blue ISG cells transduced with the indicated shRNA.  
**b.** Immunoblot of THP1-Blue ISG cells transduced with indicated shRNA and stimulated with 2′,3′-cGAMP (10 μg ml⁻¹) for 3 h and 6 h.  
**c.** Immunoprecipitation and immunoblot of THP1-Blue ISG cells transduced with indicated shRNA stimulated with 2′,3′-cGAMP (10 μg ml⁻¹) for 3 h and 6 h (upper), and immunoblot of WCLs (lower).  
**d.** Immunoprecipitation and immunoblot of shRNA-transduced THP1-Blue ISG cells stably expressing HA-Ub(K63O) for 3 h and 6 h (upper), and immunoblot of WCL (lower).  
**e.** Immunoprecipitation and immunoblot of shRNA-transduced THP1-Blue ISG cells stably expressing DAPK3(WT) or DAPK3(D161A) stimulated with 2′,3′-cGAMP (10 μg ml⁻¹) for 3 h and 6 h (upper), and immunoblot of WCL (lower).  

**f.** Immunoprecipitation and immunoblot of THP1-Blue ISG cells stimulated with 2′,3′-cGAMP (10 μg ml⁻¹) for 2.5 h and 5 h (upper), and immunoblot of WCL (lower).  
**g.** Immunoprecipitation and immunoblot of THP1-Blue ISG cells stimulated with 2′,3′-cGAMP (10 μg ml⁻¹) for 2.5 h and 5 h (upper), and immunoblot of WCL (lower).  

Anti-STING antibody (f) or anti-TBK1 antibody (g) was used for immunoprecipitation.  

Confocal fluorescence microscopy of THP1-Blue ISG stably expressing GFP-DAPK3(WT) unstimulated or stimulated with 2′,3′-cGAMP (50 μg ml⁻¹) for 3 h. Localization of GFP-DAPK3, STING and phospho-TBK1 was examined. Scale bars, 10 μm. Data in **a–h** are representative of three independent experiments.
DAPK3 promotes STING K63-linked poly-ubiquitination.

We examined the mechanism of STING activation in THP1, in which steady-state STING protein levels were unaffected by loss of DAPK3 (Fig. 5a). Upon 2′,3′-cGAMP stimulation, inducible phosphorylation of TBK1, STING and IRF3 was down-regulated by DAPK3 depletion (Fig. 5b). Coimmunoprecipitation showed that interaction between endogenous STING and TBK1 was disrupted by loss of DAPK3 (Fig. 5c), while steady-state localization and cGAMP-induced translocation of STING from endoplasmic reticulum to Golgi was unaffected (Extended Data Fig. 6a,b). Inducible K63-linked poly-ubiquitination of STING drives interaction with TBK1 (refs. 23,24). Upon 2′,3′-cGAMP stimulation, this was markedly decreased in DAPK3-depleted THP1 stably expressing HA-tagged Lys63-specific ubiquitin (HA-Ub(K63O)) (Fig. 5d).

cGAMP-induced K63-linked poly-ubiquitination of endogenous STING was also abolished in D161A-expressing THP1 (Fig. 5e).

Results demonstrate that functional DAPK3 positively regulates cGAMP-induced K63-linked poly-ubiquitination of STING.

Coimmunoprecipitation of endogenous proteins in native THP1 lysates revealed interaction between STING, DAPK3, and TBK1 or pTBK1 following cGAMP stimulation (Fig. 5f,g). In unstimulated native THP1 lysates, DAPK3 coimmunoprecipitated with TBK1 (Fig. 5g). Similarly, GFP-DAPK3 colocalized with pTBK1 and STING (Fig. 5h) or TBK1 and STING (Extended Data Fig. 6c) after cGAMP stimulation. To further probe the DAPK3–STING interaction, truncated STING proteins and STING phosphorylation mutants were assessed (Extended Data Fig. 6d). Mutations at the TBK1-acceptor sites Ser355, Ser358 and Ser366 (refs. 25,26) did not affect STING interaction with DAPK3 or TBK1 (ref. 27). However, truncated STING lacking the C-terminal region (amino acids 341–379), which interacts directly with TBK1 and is required for IRF3 activation31,32, failed to coimmunoprecipitate DAPK3 in HEK293T (Extended Data Fig. 6e). Collectively, results show that DAPK3 interacts with the C-terminal effector domain of STING and forms a tripartite complex with pTBK1 and STING after cGAMP stimulation.

Phospho-proteomic profiling uncovers DAPK3 targets.

Functional DAPK3 kinase activity is required for STING-driven IRF3 activation and IFN-β induction (Fig. 4f–h and Extended Data Figs. 3f and 5j) and cGAMP-induced STING K63-linked poly-ubiquitination (Fig. 5e). To identify DAPK3 targets, comprehensive phospho-proteomic profiling was performed using tandem mass tag (TMT)-labeling-based mass spectrometry33. THP1 cells transduced with shDAPK3, shTBK1 or shControl lentiviruses were stimulated with 2′,3′-cGAMP before quantification of phosphorylated peptides. Phospho-proteins were prioritized by identifying hypo-phosphorylation in shRNA-treated cell lysates (Fig. 6a). In total, 330 protein targets were commonly altered (301 at the same phospho-site, 29 at a different phospho-site) in both shDAPK3 cells and shTBK1 cells (Fig. 6b and Supplementary Table 2). To examine DAPK3 targets, we focused on 196 phospho-sites identified in 165 proteins demonstrating hypo-phosphorylation at DAPK3 consensus sequence R/K-X-X-S/T (ref. 34). The DAPK3-specific cluster overlapped with 16 proteins hypo-phosphorylated by the IKK consensus sequence S-X-X-S/T (ref. 35) (Fig. 6b and Supplementary Table 2).

Ingenuity Pathway Analysis (IPA) of genes in the DAPK3-specific cluster demonstrated enrichment of key regulatory kinases (for example, ERL/MAPK, mTOR and SAPK/JNK) and innate immune response genes specific for cytokine and IRF signaling (Fig. 6c). Rho signaling, actin remodeling and autophagy pathways were also highlighted, consistent with involvement of DAPK3 in these processes36,37. Collectively, results uncover a network of innate immune response proteins as substrates for DAPK3 in cGAMP-stimulated THP1.

To explore STING ubiquitination, we examined 20 candidate E3 ligases identified as potential targets for direct phosphorylation by DAPK3 (Fig. 6d and Supplementary Table 2). Genes encoding these candidates and other E3 ligases implicated in STING K63-linked poly-ubiquitination38-40 were functionally assessed in THP1 by siRNA experiments analyzing ISG reporter activity after 2′,3′-cGAMP stimulation (Extended Data Fig. 7a). Knockdown of 11 novel E3 ligase-encoding gene candidates significantly impaired ISG reporter activity, and six of these were prioritized as robust regulators (Extended Data Fig. 7b). Prioritized candidates were selected for STING ubiquitination studies, using human TRIM56 as a positive control for catalyzing K63-linked ubiquitination of human STING39. Immunoprecipitation of lysates from HEK293T transfected with Flag-tagged human STING, HA-tagged Ub (K63O) and individual V5-tagged human E3 ligases revealed that LMO7 and TRIP12 promoted K63-linked STING poly-ubiquitination (Fig. 6c).

Notably, somatic mutation and genetic alterations in DAPK3, LMO7 and TRIP12 are frequent in multiple human tumor types (Extended Data Fig. 8a–c and Supplementary Table 3). Results show that DAPK3 regulates phosphorylation of the E3 ligases LMO7 and TRIP12, which catalyze K63-linked STING ubiquitination.

LMO7 phosphorylation on S863 is critical for STING ubiquitination. We next performed in vitro kinase assays of DAPK3 and TBK1 (Extended Data Fig. 7c,d). TRIP12 was phosphorylated by DAPK3 or TBK1, and TRIM56 by TBK1. However, phosphorylation mutants of TRIM56 and TRIP12 did not affect STING–TBK1 interaction or STING K63-linked poly-ubiquitination (Extended Data Fig. 7e–h). DAPK3 phosphorylated LMO7 at S863, the site identified by phospho-proteomic profiling (Fig. 7a). TBK1 also phosphorylated LMO7 at other sites within amino acids 810–910 and amino acids 360–460 regions (Extended Data Fig. 9a). Notably, DAPK3 depletion specifically attenuated LMO7-mediated STING K63-linked ubiquitination (Extended Data Fig. 9b) and impaired STING–LMO7 interaction (Fig. 7b), consistent with inability of the phospho-deficient LMO7 S863A mutant to interact with STING (Fig. 7c). Accordingly, S863A mutation or deletion of LMO7 LIM or PDZ domains, which are responsible for protein–protein interactions, impaired STING K63-linked poly-ubiquitination (Fig. 7d,e). Thus, phosphorylation of LMO7 at S863 by DAPK3 is critical for STING–LMO7 interaction and STING K63-linked poly-ubiquitination. Depletion of LMO7 or TRIP12 in THP1 impaired cGAMP-induced K63 poly-ubiquitination of endogenous STING (Fig. 7f,g) and STING–TBK1 interaction (Extended Data Fig. 9d), resulting in impaired TBK1 activation (Extended Data Fig. 9e,f). STING activation by VACV70 or STING agonists was also abolished in LMO7- or TRIP12-depleted THP1 and HUVECs (Fig. 7h,i and Extended Data Fig. 9g–j). Data show that direct phosphorylation of K63-linked poly-ubiquitination E3 ligases such as LMO7 by DAPK3 is critical for STING interaction and ubiquitination. Collectively, results support a model whereby DAPK3 is a pleiotropic regulator of STING ubiquitination and activity in unstimulated and stimulated cells (Extended Data Fig. 10).

Discussion

IFN-β is a multifunctional cytokine that coordinates anti-tumor immunity by stimulating anti-tumor myeloid and T cell populations, tumor antigen cross-presentation and cytotoxic lymphocyte activity12. STING has emerged as a promising target for boosting IFN-β-driven anti-tumor immunity in multiple preclinical models, with STING agonists currently under active pursuit in cancer immunotherapy trials as combinatorial agents with immune checkpoint blockade13. It remains incompletely understood how tumor suppressor mutations impact STING–IFN-β responses and facilitate immune evasion. Here we show that DAPK3 is a critical activating kinase for STING via two distinct processes: (1) maintaining steady-state STING protein levels by attenuating STING K48-linked poly-ubiquitination; and (2)
Fig. 6 | Phospho-proteomic profiling uncovers DAPK3 targets. a. Heat map of phospho-sites in shControl-, shDAPK3#1- or shTBK1#1-transduced THP1-Blue ISG cells upon 2′,3′-cGAMP stimulation (10 μg ml⁻¹) for 3 h. b. Overlap of hypo-phosphorylated proteins (330) detected in shDAPK3#1 cells (420) and shTBK1#1 cells (887) (upper), and 165 proteins hypo-phosphorylated at DAPK3 consensus phosphorylation site (R/K-X-X-S/T) of which 16 proteins showed overlap hypo-phosphorylation at IKK consensus site (S-X-X-X-S/T) (lower). c, IPA of 420 genes encoding proteins hypo-phosphorylated in shTBK1#1 (887) and 165 proteins hypo-phosphorylated at DAPK3 consensus phosphorylation site (r/K-X-X-S/T), of which 16 genes encoding proteins hypo-phosphorylated at DAPK3 consensus phosphorylation site in shDAPK3#1 cells (lower). d, Heat map of E3 ligases phosphorylated at DAPK3 consensus site. Amino acids numbering is based upon UniProt ID, and S751 and S1197 of LMO7 correspond to S417 and S863, respectively, of the NCBI ID. *P < 0.05, log₂FC < −0.4 for shDAPK3#1. e, Immunoprecipitation and immunoblot of HEK293T cells transfected with plasmids encoding 3×Flag-tagged human STING, HA-tagged Ub(K63O) and V5-tagged human E3 ligase (upper), and immunoblot of WCLs (lower). Data in e are representative of four independent experiments. Statistical comparisons were conducted using two-tailed t-test (d). FC, fold change.
Fig. 7 | DAPK3 phosphorylation of LMO7 is necessary for STING K63-linked poly-ubiquitination. a. In vitro kinase assay of GST-tagged human LMO7 (amino acids 810–910). Peptides were incubated with GST-tagged DAPK3 or TBK1 in the presence of [γ-32P] ATP. b, Immunoprecipitation and immunoblot of HEK293T cells transduced with indicated shRNA before transfection with plasmids encoding HA-tagged human STING and V5-tagged human LMO7(WT) (upper), and immunoblot of WCLs (lower). c, Immunoprecipitation and immunoblot of HEK293T cells transfected with plasmids encoding HA-tagged human STING and V5-tagged human LMO7(WT) or phosphor-deficient LMO7 (S863A) (upper), and immunoblot of WCL (lower). d, Immunoprecipitation and immunoblot of HEK293T cells transfected with plasmids encoding 3× Flag-tagged human STING, V5-tagged human LMO7(WT), and V5-tagged human LMO7(S863A) (upper), and immunoblot of WCL (lower). e, Schematic representation of human LMO7 mutants. f,g, Immunoprecipitation and immunoblot of THP1-Blue ISG cells transfected with two distinct shLMO7 (f) or shTRIP12 (g) and stimulated with 2',3'-cGAMP (10 μg ml⁻¹) for 3 h and 6 h (upper), and immunoblot of WCL (lower). f, g, Immunoprecipitation and immunoblot of THP1-Blue ISG cells transfected with two distinct shLMO7 (f) or shTRIP12 (g) and stimulated with 2',3'-cGAMP (10 μg ml⁻¹) for 3 h and 6 h (upper), and immunoblot of WCL (lower). Endogenous K63-linked poly-ubiquitin chains were immunoprecipitated using K63-TUBE-Flag. h, i, RT-qPCR analysis of IFNB1 and CXCL10 in THP1-Blue ISG cells transfected with two distinct shLMO7 (h) sequences or shTRIP12 (i) sequences and stimulated with VACV70 (4 μg ml⁻¹) or c-di-GMP (10 μg ml⁻¹) for 4 h. Data in a, d, f, and g are representative of or in h and i are the mean of three independent experiments. Values represent mean ± s.d. *P < 0.05 and **P < 0.001. Statistical comparisons were conducted using two-tailed t-test (h, i). CBB, Coomassie Brilliant Blue.
DNA during neoplastic transformation, irradiation or chemotherapy. Teniposide and paclitaxel are chemo-immunotherapy agents that preferentially activate cGAS–STING signaling in tumor cells, and we show that their anti-tumor effects mainly depend upon host IFNAR signaling rather than apoptosis. Collectively, we show that productive tumor-intrinsic DAPK3–STING–IFN-β signaling is a critical component of immune surveillance.

Reduced expression of DAPK3 and STING in several human cancers occurs due to genomic hypermethylation, and low DAPK3 expression was associated with shorter survival times. We show that functional DAPK3 kinase activity is critical for the full complement of anti-tumor immunity in IFN-β-producing MCA205 tumors, and loss of DAPK3 in B16F10 tumors impaired anti-tumor effects of STING agonists and eliminated anti-tumor effects of chemo-immunotherapies. Our findings suggest that functional expression of DAPK3 in tumors is a key determinant of natural immunogenicity and cancer chemo-immunotherapy response. Mechanistic insights into DAPK3 tumor suppressor function previously focused upon proliferation and apoptosis. Here we show a mechanism whereby DAPK3 functions as a regulatory kinase of innate immune signaling that complexes with TBK1 and STING. These findings represent a paradigm shift in understanding how oncogenic mutations modulate the landscape of the tumor microenvironment to favor tumor growth by immune escape. This is relevant for developing cancer immunotherapies aimed at simultaneously boosting STING–IFN-β activation and curbing T cell exhaustion.

Kinase-active DAPK3 was essential for human STING K63-linked poly-ubiquitination, which enhances STING–TBK1 interaction and subsequent TBK1 auto-phosphorylation. Notably, human STING ubiquitination is catalyzed by several different E3 ligases, which can exert distinct effects on its activity. For example, K63-linked poly-ubiquitination of human STING by human TRIM56 is dispensable for STING trafficking from the endoplasmic reticulum to Golgi, but specifically required for STING–TBK1 interaction. In contrast, human MUL1 positively regulates STING trafficking, which indirectly affects STING–TBK1 interaction. Global phospho-proteomic profiling in THP1 cells uncovered a diverse network of phospho-proteins coregulated by DAPK3 and TBK1, consistent with our finding that these key kinases interact. We identified the DAPK3 substrates LMO7 and TRIP12 as positive regulators of cGAMP-stimulated IRF3 activation and IFN-β production. LMO7 and TRIP12 were shown to catalyze K63-linked poly-ubiquitination of STING, with DAPK3 phosphorylation of LMO7 on S863 critical for LMO7–STING interaction, STING K63-linked poly-ubiquitination and STING–TBK1 interaction. To our knowledge, this is the first demonstration that dynamic phosphorylation controls STING ubiquitination. Missense mutations and gene deletions within LMO7, TRIP12 or DAPK3 loci are frequently observed human cancers. Notably, our mechanistic studies of STING ubiquitination were performed in human cells or using human proteins, underscoring the fact that K63-linked poly-ubiquitination of human STING is well delineated compared with the mouse model. Although E3 ligases positively regulate STING in mouse cells, the relationship between STING ubiquitination and functional activity is less clear, and the role of specific E3 ligases such as TRIM56 appears to differ. It is possible that STING regulation by murine DAPK3 may involve different regulatory proteins.

Combinoprecipitation and confocal microscopy showed that DAPK3 associates with TBK1. Similar to TBK1, DAPK3 regulates IFN-β induction downstream of TLR3 and TLR4 signaling, and it is likely that DAPK3 regulates post-translational modifications of other innate signaling proteins interacting with the TBK1–DAPK3 complex. DAPK3 also regulates NFkB activation induced by STING, TLR3 and TLR4, which occurs independently of TBK1 (refs. 16,33). Further studies are required to fully delineate the pleiotropic role of DAPK3 in innate immune signaling.

Loss of DAPK3 expression correlated with reduced STING protein levels in nonhematopoietic cells. DAPK3 knockdown increased K48-linked poly-ubiquitination of STING in L929, and DAPK3 may recruit K48 de-ubiquitinate(s) (DUB) to stabilize STING. A recent study showed that iRhom2 recruits the DUB EIF3S5 to STING, leading to inhibition of proteasomal degradation. More recently, TOLLIP was identified as a key factor stabilizing STING protein in resting cells by inhibiting IRE1α-driven lysosomal degradation. However, expression of kinase-dead DAPK3 complementary DNA in DAPK3-depleted L929 and MCA205 restored STING protein expression but not responsiveness to STING agonists, supporting the notion that DAPK3 is a pleotropic regulator of STING and innate immunity.

This study uncovers a previously unknown role for the tumor suppressor kinase DAPK3 in activating the STING–IFN-β response. We uncovered a molecular driver of natural cancer immunity and response to cancer immunotherapy, based upon functional DAPK3 expression, and identified E3 ligase targets that control STING activation. Ultimately, results underscore the importance of tumor cell-intrinsic innate immunity in immune surveillance, establishing a link between oncogenic mutations and evasion of host innate immunity. Since DAPK3 expression was required for IFN-β production induced by hCMV infection, it is likely that DAPK3 also regulates anti-viral immunity, and future studies examining this are of significance.

Online content

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Methods

Cells. HUVECs (Lonza) were cultured at 37 °C and 5% CO₂ in EGM2 BulletKit medium (Lonza). NHDF from C. Benedict (La Jolla Institute for Immunology, LIJ) and HEK293T, A549, HeLa and L929 (ATCC) were cultured in DMEM and 10% heat-inactivated FBS (hi-FBS), 2 mM l-glutamine, 100 μg·ml⁻¹ penicillin-streptomycin and 10 mM HEPEs (pH 8.0). Also, 5 μg·ml⁻¹ insulin (Sigma-Aldrich) and 1 ng·ml⁻¹ bgfE (Sigma-Aldrich) were added to NHDF cultures. Mouse embryonic fibroblasts, B16F10, LLC-RFP, MC38 and MCA205 obtained from C. Benedict (LIJ), S. Schreiber (LIJ), C.C. Hedrick (LIJ), J. Scholten (National Cancer Institute, NCIC) and N.P. Restifo (NCI) respectively, were cultured in DMEM and 10% hi-FBS, 2 mM l-glutamine, 0.1 mM aminoglycosides (NEAAs), 0.1 mM sodium pyruvate and 50 μM l-gentamicin sulfate. BMDMs were cultured in RPMI1640 and 10% hi-FBS, 2 mM l-glutamine, 0.1 mM NEAAs, 55 μM 2-mercaptoethanol, 0.1 mM sodium pyruvate and cultured L929 supernatant. THP1-Blue ISG cells (Invivogen) were cultured in RPMI1640 and 10% hi-FBS, 55 μM 2-mercaptoethanol, 50 μg·ml⁻¹ l-gentamicin sulfate and 100 μg·ml⁻¹ Zeocin (Invivogen).

For nucleic acid stimulation, cells were plated at 1 × 10⁶ cells per well in 96-well plates for immunostaining, 1 × 10⁵ cells per well for RNA extraction or 5 × 10⁴ cells per well in 6-well plates for immunoblotting 1 d before stimulation. THP1-Blue ISG cells were plated at 3 × 10⁵ cells per well for RNA extraction or 2 × 10⁵ cells per well for 96-well plates for immunoblot. Nucleic acids were transacted using LyoVEC for HUVECs, Jetprime for L929-mRuby-hIRF3 or lipofectamine 2000 for THP1-Blue ISG and murine tumor lines, per manufacturer’s instructions. cGAMP stimulation was performed using lipofectamine 2000, or in cGAMP buffer containing 100 mM NaCl, 10 mM Tris-HCl, 1% BSA, 0.1% SDS, 20 mM EDTA, 1% Triton X-100 and 30% glycerol. For RNA extraction, cell strainer. Fc receptors were blocked with (FSC-A vs FSC-W, SSC-A vs SSC-W) to exclude doublets. Flow cytometry was performed as described14. ArticleS

siRNA transfection. siRNAs sequences (Dharmacon) are listed in Supplementary Table 5. siRNA pools or individual oligonucleotides were transfected into HUVECs (5 × 10⁴ cells) or L929-mRuby-hIRF3 (2 × 10⁵ cells) and plated into 6-well plates 1 d before stimulation using DharmaFECT 4 (Dharmacon). Following manufacturer’s instructions, at final concentrations of 25 nM or 40 nM, respectively. At 48 h after siRNA transfection, cells were trypsinized and replated at 1 × 10⁵ cells per well in 96-well plates for immunostaining or 1 × 10⁵ cells per well in 12-well plates for RNA extraction. Neon (Thermo Fisher Scientific) was used for BMDM and THP1-Blue ISG siRNA transfection. On day 5, BMDMs were resupplied in R buffer (5 × 10⁵ cells per well) and electroporated with 50 nM siRNA at 1,400 V/20 ms for two pulses. THP1-Blue ISG cells resuspended in R buffer (2.5 x 10⁵ cells per well) were electroporated with 100 nM siRNA at 1,625 V/10 ms for three pulses.

Total RNA extraction and RT–qPCR. Total cellular RNA was extracted using Quick-RNA Miniprep Plus Kit (Zymoresearch) and cDNA synthesized using qScript cDNA synthesis kit (Quanta), per manufacturer’s instructions. RT–qPCR was performed using CFX96 or CFX384 Touch Detection System (Bio-Rad), with Taqman Universal PCR master mix (Applied Biosystems) or FastStart SYBR Green master mix (Roche). Messenger RNA abundance of each gene was normalized to GAPDH levels for human and ACTB or B2M (encoding 18S rRNA) for mouse in Taqman assay, and ACTB/Actb for human and mouse in SYBR assay. Primer sequences are listed in Supplementary Table 6.

Immunoprecipitation and immunoblot. For whole-cell lysates, cells were lysed in 1x LifeTech lysis buffer (Cell Signaling Technology) with 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 10 mM sodium fluoride (Sigma-Aldrich). For immunoprecipitation, HEK293T or THP1-Blue ISG cells lysed in 50 mM HEPEs (pH 7.4), 100 mM KCl, 3 mM MgCl₂, 0.1 mM dithiothreitol, 85 mM sucrose, 0.2% BSA, 1 mM ATP and 0.1 mM GTP. Cells were incubated in cGAMP buffer for 15 min at 25 °C, then fresh growth medium replaced for HUVECs or overlayed for HEK293T and L929-mRuby-hIRF3. IRF3 nuclear translocation was assessed 3 h poststimulation, and RNA extraction performed 4h poststimulation.

Mouse experiments. C57BL/6 mice (WT, 800664) were obtained from the Jackson Laboratory. Ifnar1–/– mice on C57BL/6 background were obtained from S. Shresta (LJI). Mice were bred in pathogen-free conditions at the LJI vivarium. Female mice 7–10 weeks old were used and experiments conducted with blinding used age-matched and sex-matched mice. All experiments were approved by the LJF animal care committee and performed in accordance with LJF experimental guidelines.

In total, 3 × 10⁵ MCA205 or 3–7 × 10⁵ B16F10 cells were subcutaneously injected into the flank. Tumor size was measured in two dimensions using digital calipers every 2 d until a maximum of 1.0–1.5 cm³. Tumor volume was calculated using modified ellipsoid formula \[ V = \frac{L \times W^2}{2} \], where L is the widest diameter, and W the smallest diameter. For intratumor injection of 3′,3′-cGAMP (ref. 16), tumors were injected with 100 μl of PBS containing 5 μg or 10 μg of 3′,3′-cGAMP (Invivogen) with 3 μl of lipofectamine 2000 (Life Technologies) starting on day 6. PBS containing lipofectamine 2000 was used as vehicle. Also, 10 mg·l⁻¹ temopar (Sigma-Aldrich) or 10 mg·kg⁻¹ paclitaxel (Selleck Chemicals)23 was dissolved in dimethylsulfoxide, diluted with 10% dextran RL-500 (Sigma-Aldrich) or 10% dimethylsulfoxide and 4% PFA at 25 °C for 20 min and permeabilized for 15 min at 4 °C, followed by Protein G Sepharose (Sigma-Aldrich) overnight at 4 °C. Magnetic beads were incubated with STING antibody (Cell Signaling Technology, 1:50) overnight, for STING immunoblotting or 1 h for immunoprecipitation on day 6 and day 8. Further, 100 μg of anti-CD1 (PE-0035-2, BioXcell) or 100 μg of isotype control was intraperitoneally injected on days 6, 9 and 12.

Antibodies and reagents. Antibodies are listed in Supplementary Table 4. Poly (DsAT) (10B8) and MG132 (474787) were obtained from Sigma-Aldrich; VACC70 from IDT; LyoVEC, 2′,3′-cGAMP, 3′,3′-cGAMP, DMXAA, cdGMP, poly (I:C) (LMW), poly (I:C) (HMW), LPS-EB and FSL-1 from Invivogen; JetPrime from VWR; for transfections of human and mouse in SYBR assay. Primer sequences are listed in Supplementary Table 6.

Flow cytometry. Tumors were dissociated in DMEM with 400 U·ml⁻¹ collagenase type IV (Sigma-Aldrich) and 20 μg·ml⁻¹ DNase I (Worthington Biochemical) for 30 min at 37 °C. Tumor cell suspensions were washed twice with 2% FBS/0.01% sodium azide/1x PBS staining buffer, and treated with red blood cell lysis buffer (Sigma-Aldrich) for 5 min at 25 °C. Cells were washed twice with staining buffer, and filtered through 70 μm cell strainer. Cell populations were blocked with CD16/32-blocking antibody (553141, BD Biosciences) for 10 min and surface antigens stained for 30 min at 4 °C. LIVE/DEAD Fixable Blue Dead Cell Stain (L23151, Invitrogen) was used to assess viability, and forward- and side-scatter (FSC-A vs FSC-W, SSC-A vs SSC-W) to exclude doublets. Flow cytometry antibodies are listed in Supplementary Table 4. For intracellular staining, eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen) was used for Foxp3, pTBK1 and pIRF3 staining after surface staining, per manufacturer’s instructions. Fluorochrome-conjugated IgG1 isotype controls were used. The percentage of double-positive pTBK1 and pIRF3 cells. WT tumor suspensions were used as compensation controls for intracellular staining in GFP-expressing tumors. Alternatively, cells were fixed with 4% PFA at 25 °C for 20 min and permeabilized with 0.1% Triton X-100/1x PBS for 10 min before intracellular CD206 staining. Cell fluorescence was assessed using LSR-II or LSR Fortress (BD Biosciences) and data analyzed using FlowJo (TreeStar).
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and B16F10 (39). Viral titer was determined by titration in A549 or measuring with HIV Type I p24 antigen. siRNA pellets were resuspended with 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol. lysis containing Complete ULTRA Tablets (Roche) and lysed with 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM reduced glutathione. Lysates were centrifuged at 20,000 r.p.m. for 1 h at 4 °C, and the presence of 20 μM dithiothreitol lysis buffer. lysis buffer (−), GST-tagged proteins were eluted three times with 1.5 ml of 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM reduced glutathione. Enriched phospho-peptides were dried under vacuum overnight. Samples were air-dried and rehydrated in 900 μl of 1 M urea with 50 mM HEPES (pH 8.5). Phospho-proteomics. THP-1 Blue ISG cells (1x 10^6 cells per sample, n=3 biological replicates) transduced with siRNAs were stimulated with 10 μg·ml^-1 2′,3′-cGAMP for 3 h. Cells were washed three times with PBS, and pellets stored at −80 °C. mass spectrometry was performed using Orbitrap Fusion Mass Spectrometer with in-line nano-liquid chromatography. Phosphorylation was assessed by 10% SDS–PAGE and Coomassie Brilliant Blue staining followed by autoradiography.

Lentiviral transduction. HEK293T cells (6×10^5 cells per well) were transfected with 375 ng of shRNA vector and 375 ng of packaging mix. Medium was replaced 24 h post-transfection. Lenti-MEM with 10% FBS was used in place of antibiotics, and supernatants collected twice every 24 h, pooled and filtered with a 0.45-μm filter. Viral titer was determined by titration in A549 or measuring with HIV Type I p24 Antigen ELISA (20). 100 μl of 1 x 10^7 HEK293T cells per well were transduced with titrated virus, and two rounds of transduction were performed. Supernatants were collected twice with 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM reduced glutathione. Enriched phospho-peptides were dried under vacuum overnight. Samples were air-dried and rehydrated in 900 μl of 1 M urea with 50 mM HEPES (pH 8.5). Phospho-proteomics. THP-1 Blue ISG cells (1x 10^6 cells per sample, n=3 biological replicates) transduced with siRNAs were stimulated with 10 μg·ml^-1 2′,3′-cGAMP for 3 h. Cells were washed three times with PBS, and pellets stored at −80 °C. mass spectrometry was performed using Orbitrap Fusion Mass Spectrometer with in-line nano-liquid chromatography. Phosphorylation was assessed by 10% SDS–PAGE and Coomassie Brilliant Blue staining followed by autoradiography.

siRNA screening. HUEVs were transduced with siRNA pools (2007 Human siGENOME siRNA library, Dharmatag) targeting 1,001 tumor suppressor genes (38), STING or TBK1. Then, 1.25 pmol of siRNA was mixed with DharmaFect 4 (Horizon Discovery) in OptiMEM (Thermo Fisher Scientific), and aliquoted to 384-well optical imaging plates (Corning) in duplicate plates. 50,000 cells per well and incubated at 37 °C, 5% CO2 for 24 h. Medium was changed after 24 h, and cells cultured for 48 h before stimulation with poly (dA-dT)–LyoVec complexes (Invivogen) for 3 h and analysis of IFN3 nuclear translocation (16), a7iTBK1, aSTING and nontargeting nsiControl were included on each plate. Z-score was calculated as described (16). THP-1 Blue ISG cells were transduced with 100 nM siRNA using Neon. At 72 h post-transfection, cells were plated at 6×10^4 cells per well in 96-well plates and stimulated with 10 μg·ml^-1 2′,3′-cGAMP for 6 h. Culture supernatants were assessed for SEAP activity using QUNATi-Blue (Invivogen), per manufacturer’s instructions. Cell viability was assessed by CellTiter Glo (Promega) to normalize SEAP activity.

Microscopy. Fluorescence microscopy for endogenous IFN3 in HUEVs and L929-mRuby-hIFR3 was performed, as described (1). For confocal microscopy, MCA205. Blasticidin selection was performed at 5 μg/ml. THP1-Blue ISG cells (1x 10^6 cells per well) were transduced with lentivirus with 10 μg·ml^-1 2′,3′-cGAMP for 3 h. Cells were washed three times with PBS, and washed four times with PBS, and then 1x 10^5 cells were plated on glass coverslips 1 d before eGAMP stimulation. MCA205-gCas9-BL and B16F10-cGAS-Clover were incubated at 37 °C for several days at 25 μl and 0.7x 10^4 cells per well, respectively. After stimulation, cells were fixed with 4% paraformaldehyde/1x PBS for 20 min at 25 °C, washed four times with PBS and permeabilized with 0.2% Triton X-100/1x PBS for 10 min. Cells were blocked with 5% BSA in PBS for 30 min at 25 °C and stained with primary antibody in 1% donkey serum/1% goat serum/0.1% 3-3′-diaminobenzidine for 1 h at 4 °C. Cells were washed three times with PBS and incubated with secondary antibody in 1% donkey serum/1% goat serum/0.1% 3-3′-diaminobenzidine for 1 h at 25 °C. Cells were washed three times with 1x PBS and counterstained with DAPI. Slides were mounted with ProLong Gold (Life Technologies) and images acquired with an Olympus FV10i confocal microscope with a x60 objective. Colocalization was analyzed with Just Another Co-localization Plugin (JACoP) on ImageJ.
phosphopeptide level. Data from each experiment were normalized against bridge channel values normalized against median bridge channel value. Quantitation values were normalized against median quantitation values for each channel which were normalized against overall quantitation median value. A final normalization step was applied to phospho-proteomic data, wherein quantitation values for phospho-peptides were normalized against corresponding protein abundance values in proteomics data. This step is applied to prevent quantitation bias associated with increased or decreased protein abundance in absence of increased or decreased phosphorylation.

Heat map and IPA analysis. The phosphopeptide heat map was created with heatmap.2 function in ggplot2 v.3.0.1 library under RStudio v.3.6.1. logFoldChange-cutoff of 0.4 and pValue-cutoff of 0.05 in either comparison, shDAPK3#1 versus shControl or shTBK1#1 versus shControl, were used to select genes. The heat map of E3 ligases was generated with Morpheus (https://software.broadinstitute.org/morpheus/). Raw data are shown in Supplementary Table 2. QIAGEN IPA was used for gene dataset analysis.

The Cancer Genome Atlas (TCGA) data analysis. RNA-sequencing fragments per kilobase of sequence per million mapped reads (FPKM) data and survival curve data of patients with TCGA pancreatic adenocarcinoma, uterine corpus endometrial carcinoma and esophageal carcinoma were downloaded from TCGA (http://tumorsurvival.org) onto. All patients were divided into three subgroups (tertiles) according to DAPK3 expression levels and top (high) and bottom (low) tertiles were compared in survival analysis.

Cancer genomics analysis. DAPK3, LMO7 and TRIP12 alteration data and graphs were obtained from cbioPortal for Cancer Genomics (http://cbioportal.org). A TCGA case study is listed in Supplementary Table 3.

Statistical analysis. Statistical analyses were performed with Excel (Microsoft) and Prism (GraphPad). Two or three independent experiments were replicated, and error bars represent mean ± s.d. Statistical comparisons were evaluated using two-tailed Student’s t-test (two groups), with P values indicated in figure legends and source data.

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

Data availability
Screening results are presented in Supplementary Table 1 and phospho-proteomics results in Supplementary Table 2. Mass spectrometry proteome and phosphoproteome data were deposited in MassIVE (identifier PXD023639) and ProteomeXchange (identifier PXD023637). Uncropped immunoblot images are provided in the manuscript. Source data are provided with this paper. Additional data will be made available from the corresponding author upon reasonable request.

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Author contributions
M.T. designed, optimized and performed in vitro and in vivo experiments and the siRNA screen in THP1-Blue ISG cells. C.-W. J.L. optimized and performed the siRNA screen in HUVECs and generated L929 reporter cells. A.C. optimized and performed phospho-proteomics in THP1-Blue ISG cells under the supervision of D.J.G. M.S. optimized and performed mass spectrometry analysis in HEK293T and L929 lysates and supported phospho-proteomics under the supervision of M.M. F.A. provided support for bioinformatics analyses. M.J. provided support for mass spectrometry studies. S.S. provided overall direction and supervision. M.T. and S.S. wrote the manuscript with input from co-authors.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | DAPK3 is a positive regulator of STING signaling and some TLR pathways. a, Schematic representation of the RNAi screen for IRF3 nuclear translocation. b, Immunostaining of IRF3 in HUVEC stimulated with poly (dA:dT) (1 μg/ml) (right panel) for 3 h. Scale bar, 100 μm. c, d, qRT-PCR of DAPK1/Dapk1, DAPK2/Dapk2, and DAPK3/Dapk3 in (c) human and (d) mouse cell lines. #, Not detected. e, Images of IRF3 localization in L929-mRuby-hIRF3 stimulated with poly (dA:dT) (1 μg/ml) for 3 h. Scale bar, 100 μm. f, g, (f) qRT-PCR of IFNB1 and (g) immunoblot of HUVEC transfected with indicated siRNA. h, i, (h) qRT-PCR of Ifnb1 and (i) immunoblot of BMDM transfected with indicated siRNA. (f, h) Cells were stimulated with poly (dA:dT) (0.2 μg/ml) for 4 h. j, k, (j) qRT-PCR of Il6 in L929-mRuby-hIRF3 and (k) IL6 in THP1-Blue ISG transduced with indicated shRNA and stimulated as indicated in Fig. 1g–l. l, m, qRT-PCR of (l) Ifnb1 in L929-mRuby-hIRF3 and (m) IFNB1 in THP1-Blue ISG transduced with indicated shRNA and transfected with poly (I:C)(LMW) and poly (I:C)(HMW)(0.1 μg/ml for L929-mRuby-hIRF3, 0.5 μg/ml for THP1-Blue ISG). n, o, qRT-PCR of (n) IFNB1 and (o) IL6 in THP1-Blue ISG transduced with indicated shRNA and stimulated with FSL-1(100 ng/ml), naked poly (I:C)(LMW)(10 μg/ml), or LPS(100 ng/ml) for 4 h. p, q, Immunoblot of (p) THP1-Blue ISG and (q) L929-mRuby-hIRF3 transduced with indicated shRNA. Data in (b–e, g, i, p, q) are representative or (f, h, j–o) the mean of three independent experiments. Values represent mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001. Statistical comparisons were conducted using two-tailed t-test (f, h, j–o).
Extended Data Fig. 2 | Association of DAPK3 with outcomes in human cancer. Kaplan-Meier survival analysis of pancreatic adenocarcinoma, uterine corpus endometrial carcinoma, and esophageal carcinoma comparing the top (high) and bottom (low) tertiles of patients with respect to DAPK3 expression levels as reported by TCGA data portal. Statistical comparisons were conducted using two-sided log-rank test. 
Extended Data Fig. 3 | See next page for caption.
**Extended Data Fig. 3** | Teniposide and paclitaxel induce micronuclei formation and anti-tumor immunity to B16F10 tumors in a type I IFN signaling-dependent manner. 

**a, b.** (Left) Immunoblot and (right) in vitro cell growth of (a) MCA205 and (b) B16F10 transduced with indicated shRNA.

**c.** Flow cytometry of tumor-infiltrating CD8+ T cells and CD103+CD8α+DCs in MCA205 tumor suspensions isolated from WT or Ifnar1-KO mice on Day 6 (n=6 per group).

**d.** Confocal fluorescence microscopy of MCA205 stably expressing cGAS-Clover. Scale bar, 10 μm.

**e.** qRT-PCR of Ifnb1 in unstimulated MCA205 and B16F10 transduced with indicated shRNA.

**f.** (Left) Immunoblot and (right) qRT-PCR of Ifnb1 in shDapk3#1-transduced MCA205 ectopically expressing V5-tagged DAPK3(WT) or DAPK3(D161A). Cells were stimulated with 2′,3′-cGAMP, 3′,3′-cGAMP, c-di-GMP (20 μg/ml for all three agonists) or DMXAA (50 μg/ml) for 4 h.

**g.** Confocal fluorescence microscopy of B16F10 stably expressing cGAS-Clover. Cells were treated with teniposide (10 μM) for 24 h or paclitaxel (100 nM) for 72 h. Scale bar, 10 μm.

**h.** (Left) Apoptosis measured in shRNA-transduced B16F10 treated with teniposide (10 μM) for 24 h or (right) paclitaxel (100 nM) for 48 h.

**i.** Tumor volume of B16F10 subcutaneously transplanted into WT and Ifnar1-KO mice and treated with teniposide or paclitaxel (n=6 for vehicle, n=7 for teniposide and paclitaxel). Tumor size on Day 15 is represented (right panel). Data are representative (a-d, g-i) or the mean (a, b, e, f) of three independent experiments. Values represented mean ± s.d. *P<0.05, **P<0.01, and ***P<0.001. Statistical comparisons were conducted using two-tailed t-test (a-c, e, f, h, i).
Extended Data Fig. 4 | Flow cytometry gating strategy for tumor-infiltrating leukocytes. Tumor single cell suspensions were stained with different fluorophore-conjugated antibodies and analyzed by flow cytometry.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | DAPK3 does not directly phosphorylate STING or TBK1. a–d, (a) qRT-PCR of Sting and Dapk3, (b) immunoblot, (c) IRF3 nuclear translocation, and (d) p65 nuclear translocation in L929-mRuby-hIRF3 transduced with indicated shRNA stimulated with poly (dA:dT) (0.5 μg/ml) or VACV70 (2 μg/ml) for 3 h. e, Immunoblot of L929-mRuby-hIRF3 transfected with indicated siRNA. f, Immunoblot of L929-mRuby-hIRF3 transduced with indicated shRNA stimulated with VACV70 (2 μg/ml) for 2 h and 4 h. g, Immunoblot of HUVEC stably expressing VS-tagged DAPK3(D161A), DAPK3(T180A), or luciferase. Cells were infected at MOI = 5, 2, or 1. h, Immunoblot of THP1-Blue ISG stably expressing VS-tagged DAPK3(WT) or DAPK3(D161A). i, qRT-PCR of Ifnb1 in L929-mRuby-hIRF3 pre-treated with DAPK inhibitors for 3 h prior to 2′,3′-cGAMP stimulation (10 μg/ml) for 4 h. j, qRT-PCR of IFNB1 in THP1-Blue ISG pre-treated with DAPK inhibitors (50 μM) for 6 h prior to 2′,3′-cGAMP or c-di-GMP stimulation (10 μg/ml for both) for 4 h. k, l, In vitro kinase assay of (k) GST-tagged human STING C-terminus (aa 149-379) and (l) GST-tagged human TBK1(K38M). Peptides were incubated with GST-tagged DAPK3 or TBK1 in the presence of [γ-32P] ATP. Data in (b, e–h, i, l) are representative or (a, c, d, i, j) mean of three independent experiments. Values represent mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001. Statistical comparisons were conducted using two-tailed t-test (a, c, d, i, j).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | DAPK3 is not involved in STING trafficking from ER to Golgi. **a,** Confocal fluorescence microscopy of THP1-Blue ISG transduced with indicated shRNA and unstimulated or stimulated with 2′,3′-cGAMP (25μg/ml) for 3h. Scale bar, 15μm. **b,** (Upper) Co-localization of STING/Calreticulin and (lower) STING/GM130 analyzed using Image J software. Data are pooled from three independent experiments (n > 1,500 cells for unstimulated 32 images and cGAMP-stimulated 73 images). **c,** (Upper) Confocal fluorescence microscopy of THP1-Blue ISG stably expressing GFP-tagged DAPK3(WT) unstimulated or stimulated with 2′,3′-cGAMP (50μg/ml) for 3h. Localization of GFP-DAPK3, STING, and TBK1. (Lower) Co-localization of GFP-DAPK3/TBK1, GFP-DAPK3/STING, and TBK1/STING was analyzed using Image J software. Data are pooled from three independent experiments (n > 1,500 cells for unstimulated and cGAMP-stimulated 70 images). Scale bars, 15μm. **d,** Schematic representation of human STING mutants. **e,** (Upper) Immunoprecipitation and immunoblot of HEK293T transfected with plasmid encoding HA-tagged human STING (WT, 1-379), phospho-deficient mutant (3S-3A), or C-terminal deletion mutant (aa 1-340) unstimulated or stimulated with 2′,3′-cGAMP (5μg/ml) for 2h, and (lower) immunoblot of whole cell lysates (WCL). Values represented as mean ± s.d. Data in (a, c, e) are representative of three independent experiments.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Phosphorylation of TRIP12 on S312 or TRIM56 on T442 are not involved in STING K63-linked poly ubiquitination. a, Primary RNAi screen of E3 ligases in THP1-Blue ISG transfected with indicated siRNA. SEAP activity was measured after normalization with CellTiter-Glo. Black; siControl, Blue; previously reported E3 ligases for K63-linked poly-ubiquitination of STING, Red; positive control (for example siSTING1 and siTBK1). siTRIM56 value was used for determining cut-off. b, Secondary RNAi screen of E3 ligases in THP1-Blue ISG transfected with indicated siRNA. qRT-PCR of IFNB1 was performed. #; candidates for subsequent analysis. c, d, In vitro kinase assay of (c) GST-tagged human TRIP12 peptide (aa 260-360) and (d) GST-tagged human TRIM56 peptide (aa 400-500). Peptides were incubated with GST-tagged DAPK3 or TBK1 in the presence of [γ-32P] ATP. e, Schematic representation of human TRIP12 mutants. f, (Upper) Immunoprecipitation and immunoblot of HEK293T transfected with plasmids encoding HA-tagged human STING and V5-tagged human TRIP12 (WT) or phospho-deficient TRIP12 (S312A), and (lower) immunoblot of whole cell lysates (WCL). g, (Upper) Immunoprecipitation and immunoblot of HEK293T transfected with plasmids encoding 3xFlag-tagged human STING, HA-tagged Ub(K63O), and V5-tagged human TRIP12(WT), phospho-deficient TRIP12(S312A), or HECT domain-deficient TRIP12(ΔHECT), and (lower) immunoblot of WCL. h, (Upper) Immunoprecipitation and immunoblot of HEK293T transfected with plasmids encoding 3xFlag-tagged human STING, HA-tagged Ub(K63O), and V5-tagged human TRIM56(WT), phospho-deficient TRIM56(T442A), or enzyme-inactive TRIM56(C24S), and (lower) immunoblot of WCL. Data in (c, d, f-h) are representative of three independent experiments. Values represent mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001 (compared to siControl) (a, b). Statistical comparisons were conducted using two-tailed t-test (a, b).
Extended Data Fig. 8 | DAPK3, LMO7, and TRIP12 are highly mutated in human cancers. a-c. Genomic alterations of (a) DAPK3, (b) LMO7 and (c) TRIP12 in human cancers from cBioportal.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | LMO7 and TRIP12 are positive regulators of STING-IFNβ signaling in THP1 and HUVEC. a, In vitro kinase assay of GST-tagged human LMO7 (aa 360-460). Peptides were incubated with GST-tagged DAPK3 or TBK1 in the presence of [γ-32P] ATP. b, (Upper) Immunoprecipitation and immunoblot of HEK293T transduced with indicated shRNA prior to transfection with plasmids encoding 3×Flag-tagged human STING, HA-tagged Ub(K63O), and V5-tagged human LMO7(WT) and (lower) immunoblot of whole cell lysates (WCL). c, (Upper) Immunoblot of THP1-Blue ISG transduced with two distinct shLMO7 or (lower) shTRIP12 sequences. d, (Upper) Immunoprecipitation and immunoblot of THP1-Blue ISG transduced with indicated shRNA and stimulated with 2′,3′-cGAMP (10 μg/ml) for 3 h and 6 h, and (lower) immunoblot of WCL. e, f, Immunoblot of THP1-Blue ISG transduced with two distinct (e) shLMO7 or (f) shTRIP12 sequences and stimulated with 2′,3′-cGAMP (10 μg/ml) for 3 h and 6 h. g, h, Immunoblot of (g) THP1-Blue ISG and (h) HUVEC transected with indicated siRNA. i, j, qRT-PCR of IFNB1 and CXCL10 in (i) THP1-Blue ISG and (j) HUVEC transfected with indicated siRNA stimulated with VACV70 (2 μg/ml), 2′,3′-cGAMP (10 μg/ml), and c-di-GMP (10 μg/ml). Data in (a-h) are representative or (i, j) mean of three independent experiments. Values represent mean ± s.d. *P < 0.05, **P < 0.01, and ***P < 0.001. Statistical comparisons were conducted using two-tailed t-test (i, j).
Extended Data Fig. 10 | Schematic model of the DAPK3-STING axis. In unstimulated cells (L929 and MCA205), DAPK3 maintains steady-state STING levels by inhibiting STING K48-linked poly-ubiquitination and proteasome-mediated degradation. In DNA-stimulated cells (THP1), DAPK3 promotes STING activation by phosphorylating the E3 ligase LMO7 at S863, enabling LMO7-STING interaction, STING K63-linked poly-ubiquitination, and recruitment of TBK1.
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Screening results are presented in Supplementary Table 1 and phosphoproteomics results in Supplementary Table 2. Mass spectrometry proteome and phosphoproteome data were deposited in MassIVE (identifier PXD023639) and ProteomeXchange (identifier PXD023637). Source data and uncropped immunoblot images are provided in the manuscript. Additional data will be made available to the corresponding author upon reasonable request.
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- **Sample size**: For in vitro experiments, the number of samples (e.g., individual cell number for single cell assays or individual well number for bulk cell assays) was chosen to achieve 95% power for detecting statistical differences in IRF3 nuclear translocation or gene expression. For in vivo experiments, we calculated the number of samples to achieve 95% power for detecting statistical differences in tumor volume and immune features assessed by flow cytometry (n=5 or n=10 mice). For in vivo experiments, we also based sample sizes on published studies (Woo et al, Immunity 2014; Deng et al., Immunity 2014).

- **Data exclusions**: No data were excluded.

- **Replication**: All experiments were successfully replicated at least two or three times in biologically independent experiments, as indicated in figure legends.

- **Randomization**: All samples were positionally randomized on plates, microplates and tubes. Mice were randomly allocated to each experiment.

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- n/a Involved in the study
  - ☒ ChiP-seq
  - ☒ Flow cytometry
  - ☒ MRI-based neuroimaging

#### Antibodies

Antibodies used for immunoblotting and immunoprecipitation (Name, Vendor, Catalog number):

- Rabbit anti-human/mouse DAPK1, Cell Signaling Technology, 30085, dilution (1:500)
- Rabbit anti-human DAPK3, Abcam, ab210528, dilution (1:1000)
- Rabbit anti-mouse DAPK3, Life Span Biosciences, LS-C373328, dilution (1:1000)
- Rabbit anti-human LMO7, Sigma-Aldrich, LPA020923, dilution (1:1000)
- Rabbit anti-humanTRIP12, Bethyl Laboratories, A301-814A, dilution (1:1000)
- Rabbit anti-human/mouse IRF3, Cell Signaling Technology, 43005, dilution (1:1000)
- Rabbit anti-human/mouse TBK1, Cell Signaling Technology, 35045, dilution (1:100 for immunoprecipitation, 1:500 for immunoblotting)
- Rabbit anti-human/mouse TBK1, Cell Signaling Technology, 30135, dilution (1:1000)
- Rabbit anti-human/mouse STING, ABGENET, AP9747b, dilution (1:1000)
- Rabbit anti-human/mouse STING, Cell Signaling Technology, 136475, dilution (1:50 for immunoprecipitation, 1:1000 for immunoblotting)
- Rabbit anti-human/mouse phospho IRF3 (Ser396), Cell Signaling Technology, 49475, dilution (1:1000)
- Rabbit anti-human/mouse phospho TBK1 (Ser172), Cell Signaling Technology, 54835, dilution (1:1000)
- Rabbit anti-human phospho STING (Ser366), Cell Signaling Technology, 857355, dilution (1:1000)
- Rabbit anti-human phospho STING (Ser366), Cell Signaling Technology, 197815, dilution (1:1000)
- Rabbit anti-human/mouse phospho p65(Ser536), Cell Signaling Technology, 30335, dilution (1:2000)
Rabbit anti-human/mouse p65, Santa Cruz Biotechnology, sc-372, dilution(1:500)
Rabbit anti-human/mouse phospho STAT1(Tyr701), Cell Signaling Technology, 9167S, dilution (1:300)
Rabbit anti-human/mouse STAT1, Cell Signaling Technology, 9175S, dilution (1:300)
Mouse anti-human/mouse grl2A.X(Ser139), EMD Millipore, 05-636 , dilution (1:1000)
Rabbit anti-human cGAS, ABGEKT, AP10510c, dilution (1:1000)
Rabbit anti-mouse cGAS, Cell Signaling Technology, 31659S, dilution (1:1000)
Rabbit anti-human/mouse MAVS, Abcam, ab31334, dilution (1:1000)
Rabbit anti-human/mouse DDX58, Abcam, ab45428, dilution (1:1000)
Rabbit anti-human/mouse STING, Cell Signaling Technology, 5668S, dilution (1:1000)
Mouse anti-human/mouse b-actin, R&D, MA38929, dilution (1:15000)
Mouse anti-HA, Abcam, ab16918, dilution (1:100 for immunoprecipitation)
Rat anti-HA, Sigma-Aldrich, 11867423001, dilution (1:1000)
Rabbit anti-HA, Cell Signaling Technology, 3274S, dilution (1:1000)
Mouse anti-Flag, Sigma-Aldrich, F1804 (1:100 for immunoprecipitation)
Rabbit anti-Flag, Sigma-Aldrich, F7425 , dilution(1:2000)
Mouse anti-V5, Invitrogen, R960-25, dilution (1:5000 for immunoprecipitation, 1:1000 for immunoblotting)
Rabbit anti-V5, Sigma-Aldrich, VB137, dilution (1:1000)
Rabbit anti-GST[2-5], Santa Cruz Biotechnology, sc-459, dilution (1:1000)
(Secondary antibodies)
 Peroxidase-conjugated anti-rabbit IgG, Sigma-Aldrich, A0545, dilution (1:100000)
 Peroxidase-conjugated anti-mouse IgG, Sigma-Aldrich, A3682, dilution (1:10000)
 Peroxidase-conjugated anti-rat IgG, Sigma-Aldrich, A5795, dilution (1:100000)
Clean Blot IP Detection Reagent, Thermo Fisher Scientific, Pi21230, dilution (1:100)

Antibodies for immunostaining and confocal microscopy (Name, Vendor, Catalog number):
(Primary antibodies)
Rabbit anti-human/mouse IRF3, Santa Cruz Biotechnology, sc-9882, dilution (1:200)
Rabbit anti-human/mouse p65, Santa Cruz Biotechnology, sc-372, dilution (1:200)
Rabbit anti-human/mouse phospho TBK1(Ser172), Cell Signaling Technology, 5483S, dilution (1:400)
Rabbit anti-human/mouse TBK1, Abcam, ab40676, dilution (1:100)
Sheep anti-human STING, R&D, AF6516, dilution (1:50)
Rabbit anti-human/mouse/rat Caireticulin, Abcam, ab2907, dilution (1:500)
Rabbit anti-human/mouse GM130, Cell Signaling Technology, 12480S, dilution (1:5000)
Mouse anti-human/mouse grl2A.X(Ser139), EMD Millipore, 05-636, dilution (1:1000)
(Secondary antibodies)
AF568 donkey anti-sheep IgG, Invitrogen, A21099, dilution (1:1000)
AF647 goat anti-rabbit IgG, Jackson ImmunoResearch, 111-605-104, dilution (1:2000)
AF568 donkey anti-mouse IgG, Invitrogen, A10037, dilution (1:10000)

Antibodies for flow cytometric analysis (Name, Clone, Vendor, Catalog number):
Rat anti-mouse CD16/32, 2,4G2, BD Biosciences, 553141, dilution (1:200)
Rat APC-pCyt7 anti-mouse CD45, 30-F11, Biolegend, 103116, dilution (1:500)
Mouse PerCp-Cy5.5 anti-mouse NK1.1, PK136, Thermo Fisher Scientific, 45-5941-82, dilution (1:100)
Hamster FITC anti-mouse TCR beta, H57-597, Thermo Fisher Scientific, 11596181, dilution (1:200)
Rat BV421 anti-mouse CD8a, 53-6-7, Biolegend, 100737, dilution (1:100)
Rat BV605 anti-mouse CD4, RM4-5, Biolegend, 100547, dilution (1:100)
Rat BV421 anti-mouse CD4, RM4-5, Biolegend, 100543, dilution (1:100)
Rat BV550 anti-mouse CD25, PC6.2, Biolegend, 102038, dilution (1:100)
Rat APC anti-mouse/rat Foxp3, FJK-16, Thermo Fisher Scientific, 17-5773-82, dilution (1:150)
Rat PE/Dazzle594 anti-mouse ly6G, 1A8, Biolegend, 127647, dilution (1:100)
Rat BV570 anti-mouse ly6C, HK1.4, Biolegend, 128025, dilution (1:200)
Rat AF700 anti-mouse Gr-1/Ly6G/Ly6C, RB6-8C5, Thermo Fisher Scientific, 56-5931-80, dilution (1:100)
Rat PE anti-mouse/human CD11b, M1/70, Biolegend, 101207, dilution (1:1000)
Armenian Hamster FITC anti-mouse F4/80, N418, Biolegend, 117305, dilution (1:200)
Rat BV421 anti-mouse I-A/E, M5/64.15.2, Biolegend, 107631, dilution (1:500)
Rat BV605 anti-mouse CD206, C6682C, Biolegend, 141721, dilution (1:100)
Armenian Hamster APC anti-mouse CD11c, N418, Thermo Fisher Scientific, 17-0114-81, dilution (1:200)
Armenian Hamster FITC anti-mouse CD103, 2E7, Biolegend, 121439, dilution (1:200)
Rat BV605 anti-mouse/human CD11b, M1/79, Biolegend, 101207, dilution (1:1000)
Armenian Hamster BV421 anti-mouse CD11c, N418, Biolegend, 127329, dilution (1:200)
Rabbit PE control IgG, DA1E, Cell Signaling Technology, 5742S, dilution (1:500)
Rabbit PE anti-human/mouse phospho TBK1(Ser172), D52C2, Cell Signaling Technology, 13498S, dilution (1:200)
Rabbit AF647 control IgG, DA1E, Cell Signaling Technology, 2985S, dilution (1:800)
Rabbit AF647 anti-human/mouse phospho IRF3(Ser396), D601M, Cell Signaling Technology, 10327S, dilution (1:100)

Validation

All the antibodies used in this study were purchased from commercial vendors. Validation of antibodies used in this study is described in technical data sheets provided by manufacturer's websites. Primary antibodies including human/mouse DAPK3, human/mouse DAPK1, human/mouse STING, human/mouse TBK1, human LM07, human TRIP12 were validated for specificity using siRNA/shRNA depletion experiments presented in the manuscript. Antibodies for human DAPK3, DAPK1, STING, TBK1, LM07, and TRIP12 were validated in HUVEC and/or THP1 Blue-ISH. Antibodies for mouse DAPK3, DAPK1, STING, TBK1 were validated in L929-mRbU-IRF3, BMDM, MCA205, and/or B16F10.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  
HUVEC, from LONZA.  
THP1-Blue iSG, from Invivogen.  
MEF, from Dr. Christopher Benedict (UJI), originally derived from C57BL/6J available from JAX  
NHDF, from Dr. Christopher Benedict (UJI), originally from LONZA  
HEK293T, A549, HeLa, L929, from ATCC  
B16F10, from Dr. Stephen Schoenberger (UJI), originally from ATCC  
LLC-RFP, from Dr. Catherine C. Hedrick (UJI), transduced LLC cells from ATCC with RFP-expressing lentiviral vector  
MC38, from Dr. Jeffrey Schiom (NCI), established by the researcher (Robbins PF, Kantor JA, Salgaller M, Horan Hand P, Fernsten PO, Schiom J (1991) Cancer Res 51: 3657)  
MCA20S, from Dr. Nicholas O. Restifo (NCI), originally from Dr. Steven A. Rosenberg, who established the cell line (Múle JJ, Yang CJ, Lafreniere R, Shu S, Rosenberg SA (1987) J Immunol 139:285,)  
BMDMs were differentiated from BM from C57BL/6J in RPMI supplemented with L929 culture supernatants.

Authentication  
All cell lines obtained from ATCC, Invivogen, and LONZA were authenticated by the manufacturer. All cell lines used in the study were verified for purity based upon homogenous morphological characteristics using light microscopy. HUVEC, THP1-Blue iSG, MEF, NHDF, HEK293T, A549, HeLa, L929, B16F10, LLC-RFP, MC38, MCA20S and BMDM were verified using species-specific PCR primers for DAPK1, DAPK2 and DAPK3.

Mycoplasma contamination  
All primary cells and cell lines are routinely verified for negative mycoplasma contamination every 6 months. Cell lines are kept in culture for no longer than 3 months.

Commonly misidentified lines  
(See ICLAC register)  
No commonly misidentified cell lines were used in this study.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals  
C57BL/6J mice and ST/NG-gt/gt mice were purchased from the Jackson Laboratory. iNAR1-knockout mice [C57BL/6 background] were provided from Dr. Sujan Shresta. 7-10 weeks old female mice were used for this study. Mice were maintained with specific pathogen-free conditions at La Jolla Institute for Immunology. Mice were housed at an ambient temperature of approximately 22°C, a humidity of 40% to 60%, and a light/dark cycle of 12 hours.

Wild animals  
This study does not involve wild animals.

Field-collected samples  
This study does not involve field-collected samples.

Ethics oversight  
All animal work were approved by animal care committee at La Jolla Institute for Immunology, animal protocol number AP00001111, and complied with all established ethical guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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  
Tumors were dissected and dissociated with DMEM supplemented with 400 U/ml Collagenase type IV (Sigma-Aldrich) and 20 ug/ml DNase I ( Worthington Biochemical) for 30 min at 37°C. Tumor cell suspensions were washed twice with flow cytometry staining buffer (2% FBS/0.01 % sodium azide in PBS), and treated with red blood cell lysis buffer (Sigma-Aldrich) for 3 min at room temperature. Cells were washed twice with flow cytometry staining buffer, and then filtered through a 70 um cell strainer.

Instrument  
BD LSR Fortessa, BD LSR II, BD FACSAria II Cell Sorter

Software  
BD FACS Diva (Data acquisition), Flowjo version 9, Flowjo version 10 [Data analysis]

Cell population abundance  
Post-sorted samples were analyzed by FACSAria II Cell Sorter to determine the purity of the population. The population purity is at least 92%.
LIVE/DEAD Fixable Blue Dead Cell Stain (L23015, Invitrogen) was used for analysis of viability. Single cells were isolated by gating (FSC-A vs FSC-W and SSC-A vs SSC-W). Gating strategy is shown in the Extended Data Figure. 3.

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