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Peer Review Information

Journal: Nature Immnuology
Manuscript Title: The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING–IFN-β pathway
Corresponding author name(s): Sonia Sharma

Editorial Notes:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29718

Message: 26th May 2020

Dear Sonia,

We have now finished reviewing your manuscript entitled "The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING-IFNb pathway", reference number NI-A29718. Although the editors thought that the manuscript was interesting enough to send out for in-depth review, the reviewers were not in favor of publishing the paper in Nature Immunology. All three referees raised a number of concerns that would likely require substantial new experiments for publication in Nature Immunology, hence at this time we felt the best option for you for rapid publication of the study would be to try another journal with a lower bar. We are therefore returning the reviews to you with the hope that you find them useful when you prepare the paper for another journal.

You might want to consider our sister journal Nature Communications as a potential venue for the publication of these results. Nature Communications publishes high quality and influential research and across the full spectrum of the natural sciences. More information on the journal, the potential benefits of transfer and a link to transfer your paper, can be found at the bottom of this email. Please note that the editorial team at Nature Communications will consider your manuscript independently of our suggestion to transfer.

We realize that this is disappointing. I hope that you continue to consider Nature

Immunology for your results most significant for the immunology community and wish you well in your future investigations.

Kind regards & stay well,

Laurie

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Takahashi et al. investigates a new regulator of STING, called death-associated kinase 3 (DAPK3). The cGAS-STING pathway is an important innate immune pathway for detection of dsDNA. The intracellular receptor cGAS recognizes DNA from from foreign sources like bacteria and viruses or endogenous DNA (genomic, mitochondrial, micronuclear, …). Once activated, cGAS produces the second messenger cGAMP which in turn binds to STING and activating a signaling cascade resulting in phosphorylation of the transcription factor IRF3 and production of type I interferon. Besides its role in pathogen defense, the cGAS-STING pathway has been shown to play a significant role in tumor immunology as tumor-derived DNA can activate the immune system or induce tumor-intrinsic production of type I IFN and ISGs. Currently, STING agonists are tested in different pre-clinical models as they are able to boost the anti-tumor immune response and affect the efficiency of immunotherapy using checkpoint inhibitors. Thus, a profound understanding of the cGAS/STING pathways is important for the development of new cancer immunotherapies.

Here, the authors used an siRNA screening targeting ~1000 tumor suppressor genes to identify new regulators of the STING pathway. From this screening, DAPK3 emerged as the top positive regulator of STING. The authors went on to show that loss of DAPK3 impairs the dsDNA response in many different cell lines of human and murine origin. Loss of DAPK3 in MCA205 cells leads to an enhanced proliferation in an in vivo tumor model (similar to loss of STING). Furthermore, tumors lacking DAPK3 are less responsive to immunotherapy using STING agonists or checkpoint inhibitors. Moreover, the authors investigated how DAPK3 regulates the STING pathway on a molecular level. They were able to show that loss of DAPK3 leads to increase in K48 ubiquitination and proteasome degradation of STING in some cell lines. Furthermore, DAPK3 is essential for K63-linked ubiquitination of STING in THP1 cells after STING activation as well as for the interaction with TBK1. Using truncated versions of STING, it was shown that TBK1 recruits DAPK3 to the C-terminal region of STING. Finally, the group identified the E3 ligase LMO7 as target of DAPK3-dependent phosphorylation. This phosphorylation of LMO7 through DAPK3 is crucial of K63 ubiquitination of STING and its downstream activation of IRF3.

Overall, the manuscript is of high interest as it identifies a new regulator of STING and gives detailed insight on the molecular mechanism. It is well-written and the figures are

clear and sufficiently explained. However, the following issues should be addressed to further clarify the mechanisms proposed.

Major Comments

1. It would be better to show the tumor size in Figure 2c as absolute values or, if the authors want to keep a relative graph, they should put all values in relation to WT/shControl as it would be interesting to compare all shRNA groups between each other.
2. According to a PhD thesis [REDACTED] Have the authors tested the role of DAPK3 on RNA-recognition pathways or do they think that DAPK3 only regulates the STING-mediated response pathway?
3. Do the authors have absolute numbers/cell counts for the flow cytometry results in Fig 2d? In the text, it says that more Tregs and M2 MΦ are present in the knockdown tumors, yet without measuring absolute numbers, it is only allowed to state that the percentage/ratio of these cells is increased. Based on this figure, it is not known if the number of cells has changed as only the % of CD45+ is shown. If absolute numbers cannot be supplied, this section should be rephrased to make it clear that only relative numbers of cells are provided.
4. Fig 2e/f: At which time point(s) was the 3'-3' cGAMP injected? This would have a high impact on the conclusion whether or not the effectiveness of the response to the agonist is tumor-intrinsic or not. It only says "at indicated time points" in the methods section, yet the time points of injection are not indicated.
5. The phrase "Data in are representative of three independent experiments." is misleading. E.g., in Fig 1b-n: does this mean that the mean of three experiments is shown or only one representative experiment out of three? In case of the latter, please show the data as mean of all experiments and calculate the statistics based on that.
6. I agree, that the presented in vivo data shows that DAPK3 plays a role in the effectiveness of immunotherapy using 3'-3' cGAMP or checkpoint inhibitors. However, the relative reduction in tumor size in the normalized data is marginal and not as critical as described in the manuscript. For example, the authors state that "(…) DAPK3 is a key determinant of (…) efficacy of cancer immunotherapy response.". Please repharase these statements to more accurately reflect the more modest impact.
7. Does loss of DAPK3 also impair the STING-dependent activation of the NF-kB pathway or is it specific to IRF3 activation? The authors should include in vitro experiments using different DAPK3-KO/KD cell lines (murine/human, immune/tumor cells) as the authors did for the IFN/ISG response and measure the activation of NF-kB (western blot and/or IF microscopy) and expression of NF-kB-dependent cytokines (qRT-PCR and/or ELISA).

Minor Comments

1. p.4: Instead of „IRF3-STING-IFNb signaling pathway" it should read "STING-IRF3-IFNb signaling pathway".
2. Were other known regulators of STING besides IRF3 came up in the initial screening (positive controls?)?

Reviewer #2 (Remarks to the Author):

The present manuscript aims at better understanding the signaling cascade downstream of

the innate immune molecule STING. The authors propose that DAPK3, a kinase with tumor suppressive activity, acts as a regulator of STING-induced innate immune signaling. Specifically, their model implies that upon activation, STING poly-ubiquitination is faciliated by E3 ligases that are direct targets of DAPK3. This step in turn facilitates STING trafficking to the Golgi, cellular activation, and thus tumor suppression.

As detailed below there are several weaknesses in the experimental approaches that require substantial improvement to corroborate the role of DAPK3 in innate immunity. Furthermore, the study is in conflict with the generally accepted model that recruitment of TBK1 occurs at the Golgi and is in itself not required to promote intracellular STING trafficking (e.g., Ergun et al., Cell 2019; Zhang et al., Nature 2019; Dobbs et al., Cell Host Microbe 2015).

Major points:

1) Validation of DAPK3 as a specific kinase in the DNA sensing pathway: Whilst the authors are commended on using several distinct cell types to validate the role of DAPK3 in mediating responses to DNA, they do not check whether DAPK3 leaves intact or affects other innate pattern recognition receptor signaling cascades (e.g., TLR pathway, RIG-I pathway). In addition, loss-of-function experiments should include at a minimum two distinct siRNAs or shRNAs, respectively, along with a proper validation of the knockdown efficacy. These controls are incompletely provided and must be incldued.

2) Contribution of DAPK3 in cell intrinsic antitumor immunity is uncertain: The investigators propose that DAPK3 controls the immunogenicity of tumors, which is supported by changes in the infiltration of immune cells into tumors upon knockdown of DAPK3. They claim that this is mediated by tumor cell intrinsic DAPK3 being involved in mediating constant, cGAS-dependent type I IFN secretion. However, there are several uncertainties with this particular interpretation of their results: foremost there are no conclusive experiments that provide direct evidence that DAPK3 knockdown mediates type I IFN repsonses through cGAS-STING signaling. Second, it seems that the administration of STING agonists induces quite remarkable tumor control in DAPK3-suppressed tumors. Hence, at most tumor cell intrinsic DAPK3 appears to have only a minor effect on tumor cell growth and on natural or iatrogenic cancer immunotherapy.

3) Mechanism of DAPK3-dependent regulation of STING: The authors propose that in certain cell lines DAPK3 regulates steady-state STING levels through proteasomal degradation. This finding is important when considering the tumor cell effects reported above: within the responding tumor cell line (MCA205) DAPK3 considerably affects constitutive STING levels, whilst this is not the case in the non-responding tumor cell line (B16F10), respectively. Furthermore, the authors porpose that DAPK3 regulates STING trafficking and recruitment of TBK1, in a manner dependent on K63-ubiquitylation of STING. The reduced recruitment of p-TBK1 (Fig. 4c) appears to be rather a conseuqence of reduced pTBK1 levels than defects in the recruitment process per se. The impact of DAPK3 on STING trafficking is again difficult to judge, based on the usage of only one shRNA that results in modulation rather than complete inhibition. The authors must provide more thorough experimental evidence on the effect of DAPK3 on STING trafficking, e.g. by using CRISPR-KO clones of DAPK3, by multiple independent shRNA constructs, by more thorough trafficking readouts.

4) Tenary STING-TBK1-DAPK3 complex formation: A weakness of these experiments is that the formation of the complex is not thoroughly demonstrated in a native setting. Although the confocal studies presented in Fig. 5e aim into that direction, there is no quantification over a large number of cells and, more important, there appears to be co-localization between DAPK3 and pTBK1 even in unstimulated cells. This aspect needs substantial improvement by, for example, co-immunopreciptation assays in native cellular systems or by proximity-ligation assays.

5) DAPK3 activation of E3 ligases required for STING ubiquitination: Only indirect experimental evidence supports a the role of LMO7 as a DAPK3 target required for STING ubiquitination and the role of LMO7 is only supported by one (!) siRNA knockdown experiment. Is there a direct interaction between STING and LMO7, which is sensitive to DAPK3 knockdown?

Reviewer #3 (Remarks to the Author):

In this study, Takahashi et al., describe a role of DAPK3 in regulation of innate antiviral signaling and immune checkpoint blockade (ICB) therapy for cancer. Specifically, DAPK3 inhibits K48-linked ubiquitination of and stabilizes STING through an unknown mechanism, and promotes K63-linked ubiquitination of STING by recruiting and phosphorylating LMO7 and TRIP12. However, this study does not provide sufficient conceptual novelty and in vivo data to support the conclusions.

Major points
1 Cell lines were used to justify the conclusions throughout this study. The authors should generate DAPK3 KO or cKO mice to study the in vivo roles of DAPK3 in regulation of STING and STING-related antiviral and tumor immunity.

2 Even with the cell lines, siRNAs/shRNAs were used to knockdown DAPK3 or STING. The authors should use the CRISPR/Cas9 system to completely knockout the related genes, and make reconstitution assays to exclude the off-target effects. In addition, the authors should use human primary cells with siRNAs/shRNAs to determine whether a similar effects of DAPK3 on STING-related immune signaling can be observed.

3 It is very surprising that in shDAPK3 or shSTING cells, treatment of cGAMP still robustly inhibits MCA205 and B16F10 tumor growth (Fig 2f, h). DAPK3 KO and STING KO cells are strongly recommended to be used for these and other experiments. Alternatively, it is possible that cGAMP activates STING in host cells to elicit antitumor immune responses. Therefore, these experiments should be performed in DAPK3 KO or STING KO mice to exclude this possibility.

4 The authors claim the paracrine of type I IFNs from tumor cells to host cells elicits antitumor immunity and leads to tumor regression. They should examine immune cell infiltration in the tumors of wild-type and IFNAR KO host that are transplanted with DAPK3- or STING-deficient and sufficient cells.

5 The authors try to conclude a role of DAPK3 in non-immunogenic tumors in response to ICB therapy and they used B16F10 melanoma model to justify this conclusion. However,

B16 melanoma is an immunogenic tumor and ICB treatment almost completely inhibited tumor growth. The authors should try other non-immunogenic models to make such a conclusion.

6 How does DAPK3 affect K48-linked ubiquitination of STING? If DAPK3 controls basal ubiquitination and degradation of STING, the authors should examine endogenous association between DAPK3 and STING and their colocalization in subcellular compartments in unstimulated or dsDNA-transfected cells. STING is very stable with CHX treatment alone. Does STING become unstable in DAPK3-deficient cells with CHX treatment? If yes, is it rescued by MG132?

7 By using overexpression system, the authors claim that DAPK3 stabilizes STING independently of its kinase activity, and promotes K63-linked ubiquitination in a kinase activity-dependent manner. However, these experiments should be performed with DAPK3 KO cells reconstituted with WT or mutant DAPK3. In addition, the authors identified several phosphorylated sites on LMO7 and TRIP12. They should make SA or SD mutations of LMO7 and TRIP12 to examine the roles of such mutants in regulation of K63-linked ubiquitination of endogenous STING in DAPK3-sufficient and deficient cells.

Minor points
1 The knockdown efficiencies in various types of cells should be examined by Western blot assays throughout the study.
2 In Fig 2d, total cell numbers of various immune cells should be shown.
3 Which IFNAR is knocked out in the IFNAR KO mice? IFNAR1 or IFNAR2?
4 If DAPK3- or STING-knockdown affects proliferation of cells because of insufficient production of basal type I IFNs (Fig 2 and Fig S2), what is the effect by neutralizing type I IFNs with antibodies or complement of type I IFNs in the cultures of DAPK3- or STING-knockdown/KO cells?
5 In Fig 3c and 4f, the STING blot should be included in the WCL panels.
6 In Fig 4d, images with higher magnification should be shown.

Author Rebuttal to Initial comments

Reviewer 1:

1. It would be better to show the tumor size in Figure 2c as absolute values or, if the authors want to keep a relative graph, they should put all values in relation to WT/shControl as it would be interesting to compare all shRNA groups between each other.

We agree, and data in **Figure 2c** will be re-plotted to represent tumor size as absolute values, as suggested by the Reviewer.

Action item: Re-plot **Figure 2c**. Timeline: Immediate

2. According to a PhD thesis [REDACTED] Have the authors tested the role of DAPK3 on RNA-recognition pathways or do they think that DAPK3 only regulates the STING-mediated response pathway?

We assessed the role of DAPK3 in RNA-recognition pathways using HUVEC, L929 and THP1 cells. Results show that in all three cells types DAPK3 depletion has little or no significant effect upon IFNβ and IFNβ-stimulated gene expression induced by low molecular weight poly I:C (which primarily engages RIG-I), or high molecular weight poly I:C (which primarily engages MDA5). Thus, results show that DAPK3 does not directly regulate cytosolic RNA sensing pathways in three different cell types.

Regarding the PhD thesis [REDACTED]

Action item: Addition of new experimental data in HUVEC, THP1 and L929 cells demonstrating minimal or no effect of DAPK3 depletion upon IRF3 translocation or IFNβ induction upon poly I:C stimulation. Timeline: Immediate

3. Do the authors have absolute numbers/cell counts for the flow cytometry results in Fig 2d? In the text, it says that more Tregs and M2 MΦ are present in the knockdown tumors, yet without measuring absolute numbers, it is only allowed to state that the percentage/ratio of these cells is increased. Based on this figure, it is not known if the number of cells has changed as only the % of CD45+ is shown. If absolute numbers cannot be supplied, this section should be rephrased to make it clear that only relative numbers of cells are provided.

Although frequency of tumor-infiltrating cells is reported in many published studies (Sag D et al., *Nat Commun*. 2015; Carretero R et al., *Nat Immun*. 2015), the absolute number of tumor cells will be provided for **Figure 2d**, as suggested by the Reviewer.

Action item: Re-plot **Figure 2d**. Timeline: Immediate

4. Fig 2e/f: At which time point(s) was the 3'-3' cGAMP injected? This would have a high impact on the conclusion whether or not the effectiveness of the response to the agonist is tumor-intrinsic or not. It only says "at indicated time points" in the methods section, yet the time points of injection are not indicated.

We apologize for any confusion regarding the time points of cGAMP injection in **Figure 2e/f**. cGAMP was added as soon as tumors were palpable, on Day 6 post sc injection or Days 6 and 9 post sc injection.

Action item: Specify the time points of cGAMP administration to figure legend and methods sections, and include the citations for this method (Demaria et al;., *PNAS* 2015). Timeline: Immediate

5. The phrase "Data in are representative of three independent experiments." is misleading. E.g., in Fig 1b-n: does this mean that the mean of three experiments is shown or only one

representative experiment out of three? In case of the latter, please show the data as mean of all experiments and calculate the statistics based on that.

Data in **Figure 1b** can be re-calculated to reflect the mean of all three experiments, as suggested by the Reviewer.

Action item: Re-plot **Figure 1b** to reflect the mean of Fig. 1b-n experiments. Timeline: Immediate

6. I agree, that the presented in vivo data shows that DAPK3 plays a role in the effectiveness of immunotherapy using 3'-3' cGAMP or checkpoint inhibitors. However, the relative reduction in tumor size in the normalized data is marginal and not as critical as described in the manuscript. For example, the authors state that "(…) DAPK3 is a key determinant of (…) efficacy of cancer immunotherapy response.". Please re-phrase these statements to more accurately reflect the more modest impact.

The tumor-intrinsic effect elicited by administration of 3'-3'-cGAMP to MCA205 tumors (**Figure 2e-f**) is partial, likely due to the fact that 3'-3'-cGAMP is poorly cell permeable and does not efficiently enter tumor cells *in vivo* to activate tumor-intrinsic STING-IFNβ responses. To increase the efficiency of STING-IFNβ activation in B16 tumors, we can administer 3'-3'-cGAMP at an earlier timepoint, when tumors are smaller and less necrotic, as suggested by Reviewer #1. Alternatively, we can apply novel immunotherapy regimens that have recently been demonstrated to robustly engage tumor-intrinsic STING-IFNβ responses, including PARP inhibitors such as Olaparib, in combination with immune checkpoint blockade for E0771 breast cancer (Reislander et al., *Nature Communications* 2019).

Action item: Addition of new *in vivo* data using B16 and E0771 tumors to demonstrate a greater contribution of tumor-intrinsic STING-IFNβ \ to immunotherapy response. Timeline: 3-4 months

7. *Does loss of DAPK3 also impair the STING-dependent activation of the NF-kB pathway or is it specific to IRF3 activation? The authors should include in vitro experiments using different DAPK3-KO/KD cell lines (murine/human, immune/tumor cells) as the authors did for the IFN/ISG response and measure the activation of NF-kB (western blot and/or IF microscopy) and expression of NF-kB-dependent cytokines (qRT-PCR and/or ELISA).*

According to our proposed mechanism, NFkB activation should also be impaired in DAPK3-depleetd cells. We have generated data demonstrating that loss of DAPK3 impairs NFkB p65 nuclear translocation, as assessed by fluorescence imaging, and IL-6 production, as assessed by qRT-PCR. This data will be added to the manuscript, as suggested by the Reviewer.

Action item: Addition of new data demonstrating impairment of NFkB p65 nuclear translocation and cytokines (e.g. IL6) in DAPK3-deficient THP1 and L929 cells. Timeline: Immediate

Reviewer #2

1) Validation of DAPK3 as a specific kinase in the DNA sensing pathway: Whilst the authors are commended on using several distinct cell types to validate the role of DAPK3 in mediating responses to DNA, they do not check whether DAPK3 leaves intact or affects other innate pattern recognition receptor signaling cascades (e.g., TLR pathway, RIG-I pathway).

We agree with the Reviewer that it is of interest to examine whether depletion of DAPK3 affects the TLR and RIG-I innate immune pathways. As detailed in response # 2 to Reviewer 1 above, we assessed the role of DAPK3 in RNA-recognition pathways using HUVEC, L929 and THP1 cells. Results show that in all three cells types DAPK3 depletion has little or no significant effect upon IFNβ and IFNβ-stimulated gene expression induced by low molecular weight poly I:C (which primarily engages RIG-I), or high molecular weight poly I:C (which primarily engages MDA5). Thus, results show that DAPK3 does not directly regulate cytosolic RNA sensing pathways in three different cell types. Additional experiments can be performed to examine TLR ligands, and included in the manuscript.

Action item #1: Addition of new experimental data in HUVEC, THP1 and L929 cells demonstrating minimal or no effect of DAPK3 depletion upon IRF3 translocation or IFNβ induction upon poly I:C stimulation. Timeline: Immediate. Action item #2: Generation of new experimental examining the effect of DAPK3 depletion on TLR activation. Timeline: 1 month.

In addition, loss-of-function experiments should include at a minimum two distinct siRNAs or shRNAs, respectively, along with a proper validation of the knockdown efficacy. These controls are incompletely provided and must be included.

We agree with the Reviewer, and have taken pains to use multiple siRNA or shRNA sequences when examining DAPK3 loss-of-function. To increase rigor, we can provide additional corroborating data using multiple shRNAs.

Action item: Addition of new experimental data using additional multiple RNAi reagents to corroborate the data presented here. Timeline: 4 months.

2) Contribution of DAPK3 in cell intrinsic antitumor immunity is uncertain: The investigators propose that DAPK3 controls the immunogenicity of tumors, which is supported by changes in the infiltration of immune cells into tumors upon knockdown of DAPK3. They claim that this is mediated by tumor cell intrinsic DAPK3 being involved in mediating constant, cGAS-dependent type I IFN secretion. However, there are several uncertainties with this particular interpretation of their results: foremost there are no conclusive experiments that provide direct evidence that DAPK3 knockdown mediates type I IFN repsonses through cGAS-STING signaling. Second, it seems that the administration of STING agonists induces quite remarkable tumor control in DAPK3-suppressed tumors. Hence, at most tumor cell intrinsic DAPK3 appears to have only a minor effect on tumor cell growth and on natural or iatrogenic cancer immunotherapy.

To conclusively demonstrate that DAPK3 knockdown mediates type I IFN responses through cGAS-STING signaling *in vivo*, additional experimental data can be provided in DAPK3-deficient tumor cells to examine STING and TBK1 phosphorylation and ubiquitination ex vivo.

Action item #1: Addition of new data examining STING and TBK1 phsohphrylation in DAPK3-deficient tumors. Timeline: 2 months.

Regarding the partial contribution of tumor-intrinsic IFNb, this is likely due to the fact that 3'-3'-cGAMP is poorly cell permeable and does not efficiently enter tumor cells *in vivo* to activate tumor-intrinsic STING-IFNβ responses. To increase the efficiency of STING-IFNβ activation in B16 tumors, we can administer 3'-3'-cGAMP at an earlier timepoint, when tumors are smaller and less necrotic, as suggested by Reviewer #1. Alternatively, we can apply novel immunotherapy regimens that have recently been demonstrated to robustly engage tumor-intrinsic STING-IFNβ responses, including PARP inhibitors such as Olaparib, in combination with immune checkpoint blockade for E0771 breast cancer (Reislander et al., *Nature Communications* 2019).

Action item #2: Addition of new *in vivo* data using B16 and E0771 tumors to demonstrate a greater contribution of tumor-intrinsic STING-IFNβ to immunotherapy response. Timeline: 3-4 months

3*) Mechanism of DAPK3-dependent regulation of STING: The authors propose that in certain cell lines DAPK3 regulates steady-state STING levels through proteasomal degradation. This finding is important when considering the tumor cell effects reported above: within the responding tumor cell line (MCA205) DAPK3 considerably affects constitutive STING levels, whilst this is not the case in the non-responding tumor cell line (B16F10), respectively. Furthermore, the authors propose that DAPK3 regulates STING trafficking and recruitment of TBK1, in a manner dependent on K63-ubiquitylation of STING. The reduced recruitment of p-TBK1 (Fig. 4c) appears to be rather a conseuqence of reduced pTBK1 levels than defects in the recruitment process per se. The impact of DAPK3 on STING trafficking is again difficult to judge, based on the usage of only one shRNA that results in modulation rather than complete inhibition. The authors must provide more thorough experimental evidence on the effect of DAPK3 on STING trafficking, e.g. by using CRISPR-KO clones of DAPK3, by multiple independent shRNA constructs, by more thorough trafficking readouts.*

As the Reviewer correctly points out, DAPK3 regulation of STING K48-linked ubiquitination and STING protein levels occurs in a kinase-independent manner in some cell types, including HUVEC (Supplementary Fig. 1g), MCA205 (Supplementary Fig. 2a), and L929 (Figure. 3a) but not in BMDM (Supplementary Fig. 1i), B16F10 (Supplementary Fig. 2b), or THP1 (Figure. 4a). However, expression of the tumor-associated D161A kinase dead DAPK3 mutant in MCA205 cells rescued STING protein levels but did not reduce tumor growth, indicating that the kinase-

dependent mechanism involving STING activation is also at play in MCA205 as well as B16. STING activation will be directly examined in DAPK3-deficient tumors *in vivo*, as described in Point #2 to Reviewer 2.

Action item #1: Addition of new data examining STING and TBK1 phosphorylation in DAPK3-deficient tumors. Timeline: 2 months

Regarding STING trafficking, we will provide more thorough experimental evidence on the effect of DAPK3 on STING trafficking, by using examining a more thorough trafficking readout, notably biochemical fractionation.

Action item #2: Addition of new data examining STING trafficking in DAPK3-depletd cells using biochemical fractionation. Timeline: 3 months

4) Ternary STING-TBK1-DAPK3 complex formation: A weakness of these experiments is that the formation of the complex is not thoroughly demonstrated in a native setting. Although the confocal studies presented in Fig. 5e aim into that direction, there is no quantification over a large number of cells and, more important, there appears to be co-localization between DAPK3 and pTBK1 even in unstimulated cells. This aspect needs substantial improvement by, for example, co-immunopreciptation assays in native cellular systems or by proximity-ligation assays.

To increase the rigor of confocal imaging experiments in Figure 5e, we will provide quantification of DAPK3-STING-TBK1 co-localization over a large number of cells, as suggested by the Reviewer. We will also provide native co-immunoprecipitation experiments or proximity ligation assays, as suggested by the Reviewer.

Action items: (1) Addition of new data examining STING activation in DAPK3-deficient tumors. (2) Revision of results and discussion sections to clarify the multi-cellular nature of STING-IFNβ signaling in the tumor microenvironment. Timeline: 2 months.

5) DAPK3 activation of E3 ligases required for STING ubiquitination: Only indirect experimental evidence supports a role of LMO7 as a DAPK3 target required for STING ubiquitination and the role of LMO7 is only supported by one (!) siRNA knockdown experiment. Is there a direct interaction between STING and LMO7, which is sensitive to DAPK3 knockdown?

To emphasize and clarify this important point for the Reviewer, the role of LMO7 as a DAPK3 target in the STING pathway is supported by **multiple pieces of functional data**, including *in vitro* kinase assay using recombinant DAPK3 (**Figure. 7c**); IFNβ production using siRNA (**Figure. 7f, g**), and STING K63-linked ubiquitination using wild type LMO7 and phosphor-deficient LMO7 S863A mutant (**Figure. 7e**). Notably, the siLMO7 used in **Figure. 7f and 7g** corresponds to a pool of 4 different siRNAs. We wiill provide additional data examining the individual siRNAs and

shRNAs. Furthermore, we will examine the interaction between STING and LMO7 using co-immunoprecipitation.

Action items: (1) Functional analysis of LMO7 depletion using multiple RNAi. (2) co-immunoprecipitation analysis of LMO7 and STING. Timeline: 2 months

Reviewer #3

1) Cell lines were used to justify the conclusions throughout this study. The authors should generate DAPK3 KO or cKO mice to study the in vivo roles of DAPK3 in regulation of STING and STING-related antiviral and tumor immunity.

To clarify this important point for the Reviewer, our study design is focused upon specifically examining the tumor suppressor role of DAPK3 in anti-tumor innate immunity, with the objective of exploring immune evasion of natural or immunotherapy-induced immune responses in DAPK3-mutated tumors. As such, analysis of whole body DAPK3 KO or cKO mice in the context of antiviral immunity is well beyond the scope of the current study. Importantly, these studies are technically infeasible, as disruption of DAPK3 expression is lethal at both the organism level and the cellular level in primary and transformed stromal and immune cells (see response below for additional details).

Action items: Discussion of DAPK3 function in other contexts (e.g. antiviral immunity).

2) Even with the cell lines, siRNAs/shRNAs were used to knockdown DAPK3 or STING. The authors should use the CRISPR/Cas9 system to completely knockout the related genes, and make reconstitution assays to exclude the off-target effects. In addition, the authors should use human primary cells with siRNAs/shRNAs to determine whether a similar effects of DAPK3 on STING-related immune signaling can be observed.

To address this important technical concern for the Reviewer, DAPK3 whole body knockout mice are embryonic lethal (Kocher BA et al., *Mol Cancer Res*. 2014), suggesting a critical role in embryogenesis. We show that DAPK3-depleted cells demonstrate delayed *in vitro* cell growth (**Supplementary Fig 2a, 2b**), and **we were unable to generate and maintain DAPK3-deficient primary or cancer cells using the CRISPR/Cas9 system (Takahashi and Sharam, unpublished results)**. Notably, several studies demonstrate that gene knockout often fails to induce a discernible impact on cellular phenotype compared to reduction of expression of the same gene (El-Brolosy et al., PLoS Genet. 2017). Accordingly, gene mutations that truncate the encoded protein, like those generated by the CRISPR/Cas9 system, can trigger the expression of related genes, leading to functional compensation (El-Brolosy et al., *Nature*. 2019; Ma Z et al., *Nature*. 2019). Finally, a recent publication shows that transduction of Cas9 protein itself induces activation of p53 pathway (Ehache OM et al., Nat Genet. 2020). The authors also report that elevated levels of DNA repair are induced by Cas9 expression, which may affect the natural

growth and immune phenotypes cells used in this study. Therefore, we stand by the technically rigorous approach we have taken to study DAPK function.

Regarding technical rigor, we used pools of 4 different siRNAs to reduce off-targets effects and two different shRNAs for functional analysis of DAPK3 in multiple human and mouse cells. Further, we used rescue experiments with WT and kinase dead DAPK3 and small inhibitors of DAPK kinase activity to rule out off-target effects of the RNAi. These methods are well-established in the field, as we have previously reported (Sonia Sharma and Anjana Rao, *Nature Immunology* 2009).

Regarding the use of primary cells, we respectfully emphasize that we have examined primary human endothelial cells (HUVEC) and primary mouse macrophages (BMDM). Notably, the initial screen of tumor suppressor genes in Figure 1 was performed in primary human cells.

3) It is very surprising that in shDAPK3 or shSTING cells, treatment of cGAMP still robustly inhibits MCA205 and B16F10 tumor growth (Fig 2f, h). DAPK3 KO and STING KO cells are strongly recommended to be used for these and other experiments. Alternatively, it is possible that cGAMP activates STING in host cells to elicit antitumor immune responses. Therefore, these experiments should be performed in DAPK3 KO or STING KO mice to exclude this possibility.

Regarding the partial contribution of tumor-intrinsic IFNβ, this is likely due to the fact that 3'-3'-cGAMP is poorly cell permeable and does not efficiently enter tumor cells *in vivo* to activate tumor-intrinsic STING-IFNβ responses. To increase the efficiency of STING-IFNβ activation in B16 tumors, we can administer 3'-3'-cGAMP at an earlier timepoint, when tumors are smaller and less necrotic, as suggested by Reviewer #1. Alternatively, we can apply novel immunotherapy regimens that have recently been demonstrated to robustly engage tumor-intrinsic STING-IFNβ responses, including PARP inhibitors such as Olaparib, in combination with immune checkpoint blockade for E0771 breast cancer (Reislander et al., *Nature Communications* 2019).

Action item: Addition of new *in vivo* data using B16 and E0771 tumors to demonstrate a greater contribution of tumor-intrinsic STING-IFNβ to immunotherapy response. Timeline: 3-4 months

4) The authors claim the paracrine of type I IFNs from tumor cells to host cells elicits antitumor immunity and leads to tumor regression. They should examine immune cell infiltration in the tumors of wild-type and IFNAR KO host that are transplanted with DAPK3- or STING-deficient and sufficient cells.

We can provide at flow cytometric analysis of DAPK3-depleted MCA205 in IFNAR-KO mice, as suggested by the Reviewer.

Action item: Flow cytometric analysis of tumors in IFNAR KO mice. Timeline: 3-4 months.

5) The authors try to conclude a role of DAPK3 in non-immunogenic tumors in response to ICB therapy and they used B16F10 melanoma model to justify this conclusion. However, B16 melanoma is an immunogenic tumor and ICB treatment almost completely inhibited tumor growth. The authors should try other non-immunogenic models to make such a conclusion.

We acknowledge that the use of the term "*immunogenic*" among different studies is inconsistent, and can revise the phrasing to reflect "*naturally IFNβ producing*" tumors such as MCA205. Furthermore, we can include data obtained using cGAMP treatment of another "*naturally low IFNb producing*" tumor such as E0771 (breast).

Action item: Change of nomenclature. Timeline: Immediate

6) How does DAPK3 affect K48-linked ubiquitination of STING? If DAPK3 controls basal ubiquitination and degradation of STING, the authors should examine endogenous association between DAPK3 and STING and their colocalization in subcellular compartments in unstimulated or dsDNA-transfected cells. STING is very stable with CHX treatment alone. Does STING become unstable in DAPK3-deficient cells with CHX treatment? If yes, is it rescued by MG132?

Regarding STING localization, we will provide more thorough experimental evidence on the effect of DAPK3 on STING trafficking, with and without CHX by using biochemical fractionation.

Action item: Addition of new data examining STING trafficking in DAPK3-depletd cells using biochemical fractionation. Timeline: 3 months

7) By using overexpression system, the authors claim that DAPK3 stabilizes STING independently of its kinase activity, and promotes K63-linked ubiquitination in a kinase activity-dependent manner. However, these experiments should be performed with DAPK3 KO cells reconstituted with WT or mutant DAPK3. In addition, the authors identified several phosphorylated sites on LMO7 and TRIP12. They should make SA or SD mutations of LMO7 and TRIP12 to examine the roles of such mutants in regulation of K63-linked ubiquitination of endogenous STING in DAPK3-sufficient and deficient cells.

As iterated above, the CRISPR/Cas9 knockout system is not suitable for investigation of DAPK3 phenotype, because disruption of DAPK3 renders in primary and transformed stromal and immune cells inviable. We will generate stable THP1 cells constitutively expressing wild type LMO7 and phosphor-deficient LMO7-S863A to examine the role of DAPK3 on LMO7-mediated on STING ubiquitination. Notably, preliminary western blot data shows that STING-K63 ubiquitination induced by wild type LMO7 was significantly impaired in DAPK3-depleted 293T cells.

Action item: Addition of new data examining STING ub in THP1 expressing WT or S286A LMO7. Timeline: 3 months

Decision Letter, first revision:

Subject: Request for resubmission to Nature Immunology

Message: Dear Sonia,

Thank you for your letter from 18th June 2020 asking us to reconsider our decision on your manuscript, "The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING-IFNb pathway".

Thank you for supplying a detailed rebuttal to the referees comments, which as you could see were very consistent. As mentioned in the original decision letter, all three referees thought the findings were interesting but needed more mechanistic support and demonstration of the relevance of the proposed signaling pathway in the tumor setting. It seems from your response that you and your colleagues can readily address these concerns with data in hand (or reanalysis of existing datasets) and additional experiments that can be completed within 4 months.

Thus, I am happy to say that we would be willing to reconsider a revised manuscript along the outlines proposed in your rebuttal response. I'm sure, however, that you'll understand that we cannot predict the outcome of the re-review process that may in the end be the same.

Once you have made these revisions, please use the URL below to submit the revised manuscript with figures, an updated life science reporting summary and any supplemental checklists, and a point-by-point response addressing the reviewers' criticisms.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

The Editorial Policy Checklist can be found here: https://www.nature.com/documents/nr-editorial-policy-checklist.pdf

[REDACTED]

Please let us know how you wish to proceed and when we can expect your revised manuscript.

Kind regards & stay well,

Laurie

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Author Rebuttal, first revision:

Response to Reviewers, Takahashi et al.

We sincerely thank the Reviewers for thoughtful and constructive review of the manuscript by Takahashi et al. In the revised manuscript **we have taken pains to address every concern** by reformatting data and figures and adding textual clarifications, literature citations and new experimental results. These revisions **strengthen the rigor of the study** and **further support the proposed mechanism by which loss of the tumor suppressor kinase DAPK3 blunts tumor cell-intrinsic STING-IFNβ pathway activation and impairs anti-tumor immunity and response to chemo-immunotherapy.** We trust that the revised manuscript fully responds to the concerns and is both novel and rigorous.

Below is a point-by-point response addressing all concerns. Corresponding changes in the revised manuscript document are indicated there using red font.

Reviewer 1:

Major comments

1. It would be better to show the tumor size in Figure 2c as absolute values or, if the authors want to keep a relative graph, they should put all values in relation to WT/shControl as it would be interesting to compare all shRNA groups between each other.

As suggested by the Reviewer, **NEW Figure 2d** was re-plotted to show the tumor size as absolute values.

2. According to a PhD thesis [REDACTED]. *Have the authors tested the role of DAPK3 on RNA-recognition pathways or do they think that DAPK3 only regulates the STING-mediated response pathway?*

As suggested by the Reviewer, we tested the functional consequences of DAPK3 depletion on RNA recognition pathways in THP1 cells and L929 cells, finding no significant effects upon endogenous *IFNβ* mRNA expression in response to transfected low molecular weight poly I:C (which primarily engages RIG-I and to a lesser extent MDA5) or transfected high molecular weight poly I:C (which primarily engages MDA5, and to a lesser extent RIG-I) (**NEW Supplementary Figure 1l, 1m**).

Regarding the PhD thesis cited by the Reviewer, [REDACTED]

Regarding other innate immune pathways, new experimental data shows that DAPK3 depletion impairs *IFNβ* and *IL-6* mRNA expression stimulated by TLR3 (naked poly I:C) and TLR4 (LPS), but does not affect TLR2/6 signaling (diacylated lipoprotein FSL-1) (**NEW Supplementary Figure 1n, 1o**). Collectively, we report that DAPK3 does not regulate cytosolic RNA sensing through RIG-I and MDA5, and positively regulates STING and certain TLR signaling pathways.

3. Do the authors have absolute numbers/cell counts for the flow cytometry results in Fig 2d? In the text, it says that more Tregs and M2 MΦ are present in knockdown tumors, yet without measuring absolute numbers, it is only allowed to state that the percentage/ratio of these cells is increased. Based on this figure, it is not

known if the number of cells has changed as only the % of CD45+ is shown. If absolute numbers cannot be supplied, this section should be rephrased to make it clear that only relative numbers of cells are provided.

Although the frequency of tumor-infiltrating cells is routinely reported in tumor immunology studies (Sag D et al., *Nat Commun*. 2015; Carretero R et al., *Nat Immun*. 2015), as suggested by the Reviewer we provide absolute cell numbers in **Figure 2c**, **NEW Figure 3l, and NEW Supplementary Figure 2c**.

Due to significant cell death during isolation and sorting of tumor cells, we were unable to calculate absolute numbers for pTBK1+ and pIRF3+ tumor cells *in vivo* (**NEW Figure 2f, NEW Figure 3m**), and have presented these values as percentage, which is in accordance with published methodology (Ding L et al., *Cell Rep*. 2018; Pantelidou C et al., *Cancer Discov*. 2019). This is articulated in the text and figure legends.

4. Fig 2e/f: At which time point(s) was the 3'-3' cGAMP injected? This would have a high impact on the conclusion whether or not the effectiveness of the response to the agonist is tumor-intrinsic or not. It only says "at indicated time points" in the methods section, yet the time points of injection are not indicated.

We apologize for any confusion regarding timing of cGAMP injection. cGAMP was added when tumors became palpable, on Day 6 or Days 6 and 9 post-injection, which is now clearly indicated in **NEW Figure 3a** and figure legend (denoted by arrows), and in the methods section.

5. The phrase "Data are representative of three independent experiments." is misleading. E.g., in Fig 1b-n: does this mean that mean of three experiments is shown or only one representative experiment out of three? In case of the latter, please show the data as mean of all experiments and calculate statistics based on that.

As suggested by the Reviewer, all qPCR data has been re-calculated to reflect mean of three experiments along with corresponding statistical calculations. This is clarified in the figure legends.

6. I agree, that the presented in vivo data shows that DAPK3 plays a role in the effectiveness of immunotherapy using 3'-3' cGAMP or checkpoint inhibitors. However, the relative reduction in tumor size in the normalized data is marginal and not as critical as described in the manuscript. For example, the authors state that "(…) DAPKs a key determinant of (…) efficacy of cancer immunotherapy response". Please re-phrase these statements to more accurately reflect the more modest impact.

We agree that contribution of tumor-intrinsic DAPK3 and STING signaling to tumor growth and cancer immunotherapy response is a key consideration, and added new *in vivo* data and textual modifications in the revised manuscript. The anti-tumor effect of 3'-3'-cGAMP on tumors implanted in wild type host mice is partial (**NEW Figure 3a, upper panel**), likely due to the fact that 3-'3'-cGAMP is poorly permeable to the tumor *in vivo* and likely preferentially activates myeloid cells and other host immune cells in the tumor microenvironment. As a result, effects of tumor-intrinsic DAPK3 depletion or tumor-intrinsic STING depletion are partial. This partial effect is discussed in the results section for **NEW Figure 3a**.

To specifically assess contribution of tumor-intrinsic DAPK3 signaling *in vivo*, we added new experiments whereby 3'-3'-cGAMP was administered to B16F10 tumors implanted into STING-deficient ($STING^{gt/gt}$) host mice. Results show that anti-tumor activity of 3'-3'-cGAMP is entirely dependent upon expression of DAPK3 or STING in the tumor (**NEW Figure 3a, lower panel**). To further explore tumor-intrinsic DAPK3 signaling and cancer immunotherapy response, we added new experiments examining other cancer chemo-immunotherapy regimens that to specifically and robustly engage cGAS-STING-IFNβ signaling in the tumor (**NEW Figure 3b-m**). The anti-tumor effects of teniposide, a topoisomerase II inhibitor that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Chabanon M et al., *J Clin Invest*, 2019), or paclitaxel, an anti-microtubule agent that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Zierhut C et al., *Cell*. 2019; Lohard S et al., *Nat Commun*. 2020), either alone or in combination with anti-PD-1 was significantly attenuated in DAPK3-depleted tumors (**NEW Figure 3f-k**). Attenuated response was characterized by decreased numbers of tumor-infiltrating CD8+ T cells (**NEW Figure 3l**) and decreased percentage of pTBK1+ and pIRF3+ tumor cells (**NEW Figure 3m**). Additional *in vivo* data confirms that anti-tumor effects of teniposide or paclitaxel depend upon host IFNAR signaling (**NEW Supplementary Figure**

2i). Supporting *in vitro* experiments show that stimulation of B16F10 with either paclitaxel or teniposide drives co-localization of cGAS and γH2AX (**NEW Supplementary Figure 2g**), and induces *IFNβ* and *IL-6* mRNA expression in a STING or DAPK3-dependent manner (**NEW Figure 3b, 3c**). Phosphorylation of IRF3 or p65 was also significantly decreased in DAPK3-depleted B16F10 (**NEW Figure 3d, 3e**).

Together, new *in vivo* and *in vitro* data directly demonstrate that tumor-intrinsic DAPK3 expression is critical for optimal effectiveness of immunotherapy regimens that drive STING-IFNβ pathway activation in tumors, which includes partial response to 3'-3'-cGAMP and complete response to teniposide or paclitaxel. As suggested by the Reviewer, this is clearly articulated in the results section of the revised manuscript.

7. *Does loss of DAPK3 also impair the STING-dependent activation of NF-kB or is it specific to IRF3? The authors should include in vitro experiments using different DAPK3-KO/KD cell lines (murine/human, immune/tumor cells) as the authors did for the IFN/ISG response and measure activation of NF-kB (western blot and/or IF microscopy) and expression of NF-kB-dependent cytokines (qRT-PCR and/or ELISA).*

As suggested by the Reviewer, we added new *in vitro* experiments showing that depletion of DAPK3 impairs expression of the NF-kB-dependent cytokine IL-6 in L929 (mouse, stromal) and THP1 (human, immune) cells stimulated with poly (dA:dT), VACV70 or 2'3'-cGAMP (**NEW Supplementary Fig 1j, 1k**), and reduces p65 nuclear translocation in L929 cells stimulated with poly (dA:dT) or VACV70, as determined by IF microscopy (**NEW Supplementary Figure 4d**).

Minor comments

1. *p.4: Instead of IRF3-STING-IFNb signaling pathway it should read STING-IRF3-IFNb signaling pathway.*

This has been corrected in the revised manuscript.

2. *Were other known regulators of STING besides IRF3 came up in the initial screening (positive controls)?*

Other than IRF3, siRNAs targeting STING and TBK1 were included as positive controls in the screen which is clarified in the methods section.

Reviewer #2

Major comments

1) Validation of DAPK3 as a specific kinase in the DNA sensing pathway: Whilst the authors are commended on using several distinct cell types to validate the role of DAPK3 in mediating responses to DNA, they do not check whether DAPK3 leaves intact or affects other innate pattern recognition receptor signaling cascades (e.g., TLR pathway, RIG-I pathway).

We agree with the Reviewer that it is important to examine how depletion of DAPK3 affects other innate pattern recognition receptor signaling cascades. As detailed in response #2 to Reviewer 1, we added new data demonstrating that depletion of DAPK3 does not affect *IFNβ* or *IL6* mRNA expression induced by transfected poly I:C ligands for RIG-I and MDA5 in L929 and THP1 (**NEW Supplementary Figure 1l, 1m**).

New data shows that DAPK3 depletion impairs *IFNβ* and *IL-6* mRNA expression induced by TLR3 (non-transfected poly I:C) and TLR4 (LPS), but does not affect TLR2/6 signaling (FSL-1) (**NEW Supplementary Figure 1n, 1o**). Collectively, data show that DAPK3 does not regulate cytosolic RNA sensing through RIG-I and MDA5, and is a positive regulator of STING and certain TLR signaling pathways.

In addition, loss-of-function experiments should include at a minimum two distinct siRNAs or shRNAs, respectively, along with a proper validation of the knockdown efficacy. These controls are incompletely provided and must be included.

As suggested by the Reviewer, we added new results for two distinct siRNAs or shRNAs targeting DAPK3 (mouse or human), STING (mouse or human), TBK1 (mouse or human), LMO7 (human) and TRIP12 (human). Importantly, protein knockdown efficiency for all siRNAs or shRNAs is provided by western blotting

in the relevant cell type in **Supplementary Figure 1g and Supplementary Figure 7h** (HUVEC); **Supplementary Figure 1i** (BMDM); **Supplementary Figure 2a** (MCA205); **Supplementary Figure 2b** (B16F10); **Figure 5a, NEW Supplementary Figure 1p, and Supplementary Figure 7c, 7g** (THP1); **Figure 4a, NEW Supplementary Figure 1q, and Supplementary Figure 4b, 4e** (L929).

2) Contribution of DAPK3 in cell intrinsic antitumor immunity is uncertain: The investigators propose that DAPK3 controls the immunogenicity of tumors, which is supported by changes in the infiltration of immune cells into tumors upon knockdown of DAPK3. They claim that this is mediated by tumor cell intrinsic DAPK3 being involved in mediating constant, cGAS-dependent type I IFN secretion. However, there are several uncertainties with this particular interpretation of their results: foremost there are no conclusive experiments that provide direct evidence that DAPK3 knockdown mediates type I IFN responses through cGAS-STING signaling. Second, it seems that the administration of STING agonists induces quite remarkable tumor control in DAPK3-suppressed tumors. Hence, at most tumor cell intrinsic DAPK3 appears to have only a minor effect on tumor cell growth and on natural or iatrogenic cancer immunotherapy.

We agree with the Reviewer that the contribution of tumor-intrinsic DAPK3 to STING signaling and tumor control is a key consideration, and include new *in vivo* and *in vitro* data as well as text modifications in the revised manuscript to support the assertion that DAPK3-suppressed tumors are impaired in intrinsic STING-IFNβ pathway activation, anti-tumor immunity and response to chemo-immunotherapy.

The revised manuscript provides **multiple lines of direct and conclusive experimental evidence to demonstrate that DAPK3 knockdown mediates IFNβ responses through the cGAS-STING pathway in cancer cells and tumors**. First, specific depletion of DAPK3, using a pool of four distinct siRNAs or two distinct shRNAs, impairs endogenous *IFNβ* mRNA induction in response to STING-specific agonists (e.g. VACV70 DNA, HCMV, 2'3'-cGAMP) in a multitude of mouse and human primary and transformed cells, including MCA205 and B16F10 tumor cells (**Figures 1d-n**). Second, depletion of DAPK3 in MCA205 tumors and B16F10 tumors treated with teniposide or paclitaxel, which are IFNβ-producing tumors in which cGAS-STING signaling is specifically activated (Chabanon M et al., *J Clin Invest*, 2019; Zierhut C et al., *Cell*. 2019; Lohard S et al., *Nat Commun*. 2020) (**NEW Figure 3b, 3c**, **Supplementary Figure 2d-g**), phenocopy the specific changes in tumor growth and tumor immunology observed in STING-depleted tumors, including reduced infiltration of CD8+ T cells, CD103+CD8α+DCs, or NK cells (**Figure 2c, NEW Figure 3l**), and increased infiltration of CD4+ Treg (**Figure 2c**). Third, *ex vivo* intracellular flow cytometry analysis of tumor cells demonstrates that percentage of pTBK1+ and pIRF3+ tumor cells in untreated MCA205 tumors or B16F10 tumors treated with teniposide or paclitaxel is attenuated by depletion of DAPK3 (**NEW Figure 2f, NEW Figure 3m**). Fourth, we confirmed cGAS activation in unstimulated MCA205 cells and teniposide- or paclitaxel-treated B16F10 cells by demonstrating basal (MCA205) or inducible (B16F10) co-localization with γ-H2AX (**Supplementary Figure 2d and NEW Supplementary Figure 2g**).

Regarding tumor control of DAPK3-suppressed tumors, the Reviewer is correct that anti-tumor effect of 3'-3'-cGAMP in B16F10 tumors is partial (**NEW Figure 3a, upper panel**), likely due to the fact that 3'3'-cGAMP is poorly permeable to tumors *in vivo* and preferentially activates tumor-infiltrating myeloid cells and other host immune cells in the tumor microenvironment.

To specifically assess contribution of tumor-intrinsic DAPK3 signaling *in vivo*, we added new experiments whereby 3'-3'-cGAMP was administered to B16F10 tumors implanted into STING-deficient ($STING^{gt/gt}$) host mice. Results show that anti-tumor activity of 3'-3'-cGAMP is entirely dependent upon expression of DAPK3 or STING in the tumor (**NEW Figure 3a, lower panel**). To further explore tumor-intrinsic DAPK3 signaling and cancer immunotherapy response, we added new experiments examining other cancer chemo-immunotherapy regimens that to specifically and robustly engage cGAS-STING-IFNβ signaling in the tumor (**NEW Figure 3b-m**). The anti-tumor effects of teniposide, a topoisomerase II inhibitor that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Chabanon M et al., *J Clin Invest*, 2019), or paclitaxel, an anti-microtubule agent that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Zierhut C et al., *Cell*. 2019; Lohard S et al., *Nat Commun*. 2020), either alone or in combination with anti-PD-1 was significantly attenuated in DAPK3-depleted tumors (**NEW Figure 3f-k**). Attenuated response was characterized by decreased numbers of tumor-infiltrating CD8+ T cells (**NEW Figure 3l**) and decreased percentage of pTBK1+ and pIRF3+ tumor cells (**NEW Figure 3m**). Additional *in vivo* data confirms that anti-

tumor effects of teniposide or paclitaxel depend upon host IFNAR signaling (**NEW Supplementary Figure 2i**). Supporting *in vitro* experiments show that stimulation of B16F10 with either paclitaxel or teniposide drives co-localization of cGAS and γH2AX (**NEW Supplementary Figure 2g**), and induces *IFNβ* and *IL-6* mRNA expression in a STING or DAPK3-dependent manner (**NEW Figure 3b, 3c**). Phosphorylation of IRF3 or p65 was also significantly decreased in DAPK3-depleted B16F10 (**NEW Figure 3d, 3e**).

Together, new *in vivo* and *in vitro* data directly demonstrate that tumor-intrinsic DAPK3 expression is critical for optimal effectiveness of immunotherapy regimens that drive STING-IFNβ pathway activation in tumors, which includes partial response to 3'-3'-cGAMP and complete response to teniposide or paclitaxel. This is clearly articulated in the results section of the revised manuscript.

3) Mechanism of DAPK3-dependent regulation of STING: The authors propose that in certain cell lines DAPK3 regulates steady-state STING levels through proteasomal degradation. This finding is important when considering the tumor cell effects reported above: within the responding tumor cell line (MCA205) DAPK3 considerably affects constitutive STING levels, whilst this is not the case in the non-responding tumor cell line (B16F10), respectively.

As the Reviewer iterates, DAPK3-dependent regulation of steady-state STING levels and STING K48-linked ubiquitination occurs in a kinase-independent manner in certain cell types, including HUVEC (**Supplementary Figure 1g**), MCA205 (**Supplementary Figure 2a**), and L929 (**Figure. 4a**). In contrast, DAPK3 depletion does not affect steady-state STING levels in other cell types, including BMDM (**Supplementary Figure 1i**), B16F10 (**Supplementary Figure 2b**), or THP1 (**Figure. 5a**). Notably, expression of a tumor-associated, kinase dead mutant of DAPK3 (D161A) in MCA205 cells restored steady-state STING levels to wild type levels (**Supplementary Figure 2f)** however STING-dependent baseline and stimulated expression of IFNβ were still impaired (**Supplementary Figure 2f).** Accordingly, growth of MCA205 D161A tumors was accelerated *in vivo* (**Figure 2e**). Results suggest that in certain cell types such as MCA205, DAPK3 plays a kinase-independent role in regulating steady-state STING levels and a kinase-dependent role in regulating agonist-induced STING activation. Importantly, regardless of whether effects upon steady-state STING levels or STING activation are at play, MCA205 and B16F10 tumors are significantly impacted by loss of DAKP3 expression *in vivo*, as detailed in response #2 above.

Furthermore, the authors propose that DAPK3 regulates STING trafficking and recruitment of TBK1, in a manner dependent on K63-ubiquitylation of STING. The reduced recruitment of p-TBK1 (Fig. 4c) appears to be rather a consequence of reduced pTBK1 levels than defects in the recruitment process per se.

Regarding STING trafficking, please see detailed response below. Regarding pTBK1 recruitment to STING, as suggested by the Reviewer we performed additional co-immunoprecipitation assays in THP1 cell lysates using anti-STING antibody to examine endogenous STING interactions in a native setting. Using this methodology, we show that loss of DAPK3 significantly impaired cGAMP-induced co-immunoprecipitation of *total TBK1* not pTBK1 (**NEW Figure 5c**), and replaced initial data in THP1 cells over-expressing STING-HA with new data examining interactions of the endogenous proteins in a native setting.

Regarding regulation of K63-linked ubiquitination of STING by DAPK3, we provide multiple lines of direct evidence demonstrating that DAPK3, LMO7 and TRIP12 promote K63-linked poly-ubiquitination of STING (**Figure 5d, NEW Figure 7f, 7g**), and LMO7-mediated K63-linked poly-ubiquitination of STING depends on DAPK3 (**NEW Supplementary Figure 7b**). Further, we find that DAPK3-mediated phosphorylation of LMO7 at S863, the site identified by phosphoproteomic analysis (**Figure 6d**), is required for STING-LMO7 interaction (**NEW Figure 7b, c**) and STING K63-linked poly-ubiquitination (**NEW Figure 7d**). Mechanistic studies were performed in human THP1 cells, due to the fact that K63-linked poly-ubiquitination of human STING is well delineated compared to mouse STING (Tsuchida T et al., *Immunity*. 2010; Zhang J et al., *J Biol Chem*. 2012). Although it has been reported that certain E3 ligases positively regulate STING in MEFs, murine macrophages and dendritic cells (Tsuchida T et al., *Immunity*. 2010; Yang I et al., *Nat Commun*. 2018), the relationship between murine STING K63-linked ubiquitination and the functional activity of STING is unclear (Ni G et al., *Sci Immunol*. 2017), and regulation of STING by ubiquitination may differ in mouse cells. However, we clearly show that kinase-active DAPK3 is critical for agonist-induced STING activation in murine MCA205 (**NEW Supplementary Figure 2f**) and L929 cells in addition to HUVEC, and THP1 (**Figure**

4f-h) independently of steady-state STING levels, indicating that DAPK3 kinase activity is critical for activation of both human and mouse STING. This is clarified in the discussion of the revised manuscript.

The impact of DAPK3 on STING trafficking is again difficult to judge, based on the usage of only one shRNA that results in modulation rather than complete inhibition. The authors must provide more thorough experimental evidence on the effect of DAPK3 on STING trafficking, e.g. by using CRISPR-KO clones of DAPK3, by multiple independent shRNA constructs, by more thorough trafficking readouts.

Regarding the impact of DAPK3 on STING trafficking, we sincerely thank the Reviewer for bringing an important technical consideration to our attention. As suggested by the Reviewer, we revised the experimental methodology to thoroughly and rigorously assess STING trafficking at the single-cell level, by examining 2 distinct shRNAs targeting DAPK3, more efficiently applying cGAMP in permeabilized cells by overlaying cGAMP in buffer containing digitonin and including >1,500 cells in the analysis. Using these improved and rigorous methodologies, we find that STING trafficking is not significantly affected by depletion of DAPK3 (**NEW Supplementary Figure 5a, 5b**). This is clarified in the results and methods section of the revised manuscript.

Regarding the use of CRISPR-KO clones of DAPK3, previous studies show that DAPK3 whole body knockout is embryonic lethal in mice (Kocher BA et al., *Mol Cancer Res*. 2014), suggesting a critical role for DAPK3 in development. Our data show that DAPK3-depleted MCA205 and B16F10 cells demonstrate delayed *in vitro* growth (**Supplementary Figure 2a, 2b**), and most importantly we were consistently unable to generate and maintain DAPK3-deficient lines using the CRISPR/Cas9 system in multiple primary cells and cancer cells despite testing several distinct sgRNA sequences and delivery methods (Takahashi and Sharma, *unpublished results*). This is clarified in the results and discussion section of the revised manuscript. Notably, several studies demonstrate gene disruption often fails to induce functional impact on cellular phenotype compared to gene depletion (El-Brolosy et al., *PLoS Genet*. 2017). Accordingly, gene mutations that truncate the encoded protein can trigger expression of related genes, leading to functional compensation (El-Brolosy et al., *Nature*. 2019; Ma Z et al., *Nature*. 2019). Finally, a recent publication shows that expression of Cas9 protein activates the p53 pathway and triggers elevated DNA repair (Haapaniemi E et al., *Nat Med*. 2018; Bowden AR et al., *Elife*. 2020; Enache OM et al., *Nat Genet*. 2020), which might affect growth and immune phenotypes in this study.

To minimize potential off-targets effects of RNAi, we applied rigorous methods to validate experimental observations in DAPK3 knockdown cells using 1) multiple RNAi sequences (e.g. pool of 4 siRNAs and 2 distinct shRNAs); 2) cDNA rescue experiments with wild type or kinase dead DAPK3; 3) pharmacological inhibitors of DAPK3 kinase activity. These are the established methods for validating RNAi experiments to analyze immunological phenotypes, as we previously reported (Sonia Sharma and Anjana Rao, *Nature Immunology* 2009).

4) Ternary STING-TBK1-DAPK3 complex formation: A weakness of these experiments is that the formation of the complex is not thoroughly demonstrated in a native setting.

As suggested by the Reviewer, to demonstrate ternary *STING-TBK1-DAPK3* complex formation in a native setting we performed co-immunoprecipitations of endogenous STING using anti-STING antibody. Using this methodology, we detected interaction between endogenous STING and DAPK3 in unstimulated L929 cells (**NEW Figure 4d**), and ternary complex formation with TBK1 after 2'3'-cGAMP stimulation in L929 and THP1 cells (**NEW Figure 4d, 5f, 5g**).

Although the confocal studies presented in Fig. 5e aim into that direction, there is no quantification over a large number of cells and, more important, there appears to be co-localization between DAPK3 and pTBK1 even in unstimulated cells. This aspect needs substantial improvement by, for example, co-immunopreciptation assays in native cellular systems or by proximity-ligation assays.

As suggested by the Reviewer, we strengthened the rigor of interaction studies by providing several new pieces of experimental data to support complex formation between DAPK3 and TBK1 or STING in

unstimulated cells and stimulated cells. First, we provide new co-immunoprecipitation experiments of endogenous STING in a native setting in L929 and THP1 cells, as described above (**NEW Figure 4d and 5f**). Second, we confirmed co-localization of GFP-DAPK3 and TBK1 in unstimulated and stimulated THP1 cells (**NEW Supplementary Figure 5c**), supporting results obtained in prior interaction experiments that were performed in HEK293T cells over-expressing tagged DAPK3 and TBK1. Third, we provide additional images of DAPK3-STING-pTBK1 co-localization (**NEW Figure 5h**) and DAPK3-STING and DAPK3-TBK1 co-localization (**NEW Supplementary Figure 5c**) with higher magnification. Fourth, we provide quantification of DAPK3-STING and DAPK3-TBK1 co-localization (**NEW Supplementary Figure 5c**).

5) DAPK3 activation of E3 ligases is required for STING ubiquitination: Only indirect experimental evidence supports a role of LMO7 as a DAPK3 target required for STING ubiquitination and the role of LMO7 is only supported by one (!) siRNA knockdown experiment. Is there a direct interaction between STING and LMO7, which is sensitive to DAPK3 knockdown?

In the revised manuscript, the role of LMO7 as a phosphorylation target of DAPK3 involved in STING K63-linked ubiquitination is now supported by multiple pieces of direct evidence and functional data: 1) *in vitro* kinase assays demonstrating that recombinant DAPK3 phosphorylates recombinant LMO7 (**Figure 7a**) at DAPK3-specific consensus sites that were initially identified in unbiased phosphor-proteomic profiling (**Figure 6d**); 2) reduced expression of *IFNβ* mRNA in LMO7-depleted HUVEC or THP1 treated with STING pathway agonists (**Supplementary Figure 7g-j**); 3) reduced K63-linked ubiquitination of STING in HEK293T cells using the phospho-deficient LMO7 S863A mutant compared to wild type LMO7 (**Figure 7d**); 4) reduced K63-linked ubiquitination of endogenous STING, STING-TBK1 interaction, and subsequent phosphorylation of TBK1, STING, and IRF3 in a native setting in LMO7-depleted THP1 cells or TRIP12-depleted THP1 cells (**NEW Figure 7f, 7g, Supplementary Figure 7d-f**); notably, siLMO7 and siTRIP12 used in **NEW Supplementary Figure 7g-j** correspond to a pool of 4 distinct siRNA sequences, and we also provide new data using two distinct shRNAs targeting LMO7 or TRIP12 to examine agonist-induced IFNβ induction, endogenous STING K63-linked ubiquitination, and TBK1 activation (**NEW Figure 7f-i, NEW Supplementary Figure 7e, 7f**); 5) New data show that LMO7-induced K63-linked ubiquitination of STING depends upon expression of DAPK3 but not TBK1 (**NEW Supplementary Figure 7b**), which is in line with published reports that TBK1 depletion does not impair STING K63-linked ubiquitination (Prabakaran T et al., *EMBO J.* 2018); 6) regarding direct interaction between STING and LMO7, we provide experimental data demonstrating a STING-TRIP12 interaction (**NEW Supplementary Figure 6f**) and STING-LMO7 interaction (**NEW Figure 7b**) in HEK293T cells. Notably, depletion of DAPK3 significantly impaired the STING-LMO7 interaction (**NEW Figure 7b**), and the phospho-deficient LMO7 S863A mutant lost the capacity to interact with STING (**NEW Figure 7c**). These results show that DAPK3-mediated phosphorylation of LMO7 is critical for STING-LMO7 interaction and subsequent K63-linked poly-ubiquitination of STING.

Reviewer #3

Major comments

1) Cell lines were used to justify the conclusions throughout this study. The authors should generate DAPK3 KO or cKO mice to study the in vivo roles of DAPK3 in regulation of STING and STING-related antiviral and tumor immunity.

To clarify this key point for the Reviewer, the initial genetic screen of tumor suppressor genes and downstream studies of DAPK3, which emerged from the TSG screen, are conceptually focused upon tumor suppressor activity. Thus, we focused *in vivo* studies to study immune evasion in DAPK3-deficient tumors and rigorous *in vivo* analysis of anti-viral immunity is well beyond the scope of the current study, but an interesting topic for a future study. Notably, studies of DAPK3 KO or cKO mice are technically infeasible at this time, as disruption of DAPK3 expression is lethal at the organism level and in both stromal cells and immune cells (*see response #2 below for additional details*).

2) Even with the cell lines, siRNAs/shRNAs were used to knockdown DAPK3 or STING. The authors should use the CRISPR/Cas9 system to completely knockout the related genes, and make reconstitution assays to exclude the off-target effects. In addition, the authors should use human primary cells with siRNAs/shRNAs to determine whether a similar effect of DAPK3 on STING-related immune signaling can be observed.

Regarding use of CRISPR-KO clones of DAPK3, previous studies show that DAPK3 whole body knockout is embryonic lethal in mice (Kocher BA et al., *Mol Cancer Res*. 2014), suggesting a critical role in development. Our data confirm that DAPK3-depleted MCA205 and B16F10 cells demonstrate delayed *in vitro* growth (**Supplementary Figure 2a, 2b**), and most importantly we were consistently unable to generate and maintain DAPK3-deficient lines using the CRISPR/Cas9 system in multiple primary cells and cancer cells despite testing several distinct sgRNA sequences and delivery methods (Takahashi and Sharma, *unpublished results*). Notably, numerous studies demonstrate that gene disruption sometimes fails to induce a discernible functional impact compared to gene depletion (El-Brolosy et al., *PLoS Genet*. 2017). Accordingly, gene mutations that truncate the encoded protein can trigger related genes, leading to functional compensation (El-Brolosy et al., *Nature*. 2019; Ma Z et al., *Nature*. 2019). Finally, a recent publication shows that expression of Cas9 protein activates the p53 pathway and triggers elevated DNA repair (Haapaniemi E et al., *Nat Med*. 2018; Bowden AR et al., *Elife*. 2020; Enache OM et al., *Nat Genet*. 2020), which might affect growth and immune phenotypes in this study.

To minimize potential effects of RNAi off-targets, we applied rigorous methods to validate experimental observations in DAPK3 knockdown cells using 1) multiple RNAi sequences (e.g. pool of 4 siRNAs and 2 distinct shRNAs); 2) cDNA rescue experiments with wild type or kinase dead DAPK3; 3) pharmacological inhibitors of DAPK3 kinase activity. These are established methods for validating RNAi experiments to analyze immunological phenotypes, as we previously reported (Sonia Sharma and Anjana Rao, *Nature Immunology* 2009).

Regarding primary cells, we include analysis of primary human endothelial cells (HUVEC) and primary mouse macrophages (BMDM) in **Supplementary Figure 1f-i**. Notably, the initial loss-of-function screen in **Figure 1a** identifying DAPK3 as a regulator of DNA-induced IRF3 activation was performed in HUVEC.

3) It is very surprising that in shDAPK3 or shSTING cells, treatment of cGAMP still robustly inhibits MCA205 and B16F10 tumor growth (Fig 2f, h). DAPK3 KO and STING KO cells are strongly recommended to be used for these and other experiments. Alternatively, it is possible that cGAMP activates STING in host cells to elicit antitumor immune responses. Therefore, these experiments should be performed in DAPK3 KO or STING KO mice to exclude this possibility.

Regarding DAPK3 KO cells, please refer to response #2, above. We agree with the Reviewer that the contribution of tumor-intrinsic DAPK3 and STING signaling to tumor growth and cancer immunotherapy response is a key consideration, and have included new *in vivo* data and *in vitro* results as well as text modifications to address this important point.

Regarding tumor control of DAPK3-suppressed tumors, the Reviewer is correct that anti-tumor effect of 3'-3'-cGAMP in B16F10 tumors is partial (**NEW Figure 3a, upper panel**), likely due to the fact that 3'3'-cGAMP is poorly permeable to tumors *in vivo* and preferentially activates tumor-infiltrating myeloid cells and other host immune cells in the tumor microenvironment.

To specifically assess contribution of tumor-intrinsic DAPK3 signaling *in vivo*, we added new experiments whereby 3'-3'-cGAMP was administered to B16F10 tumors implanted into STING-deficient ($STING^{gt/gt}$) host mice. Results show that anti-tumor activity of 3'-3'-cGAMP is entirely dependent upon expression of DAPK3 or STING in the tumor (**NEW Figure 3a, lower panel**). To further explore tumor-intrinsic DAPK3 signaling and cancer immunotherapy response, we added new experiments examining other cancer chemo-immunotherapy regimens that to specifically and robustly engage cGAS-STING-IFNβ signaling in the tumor (**NEW Figure 3b-m**). The anti-tumor effects of teniposide, a topoisomerase II inhibitor that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Chabanon M et al., *J Clin Invest*, 2019), or paclitaxel, an anti-microtubule agent that specifically activates tumor-intrinsic cGAS-STING-IFNβ signaling (Zierhut C et al., *Cell*. 2019; Lohard S et al., *Nat Commun*. 2020), either alone or in combination with anti-PD-1 was significantly attenuated in DAPK3-depleted tumors (**NEW Figure 3f-k**). Attenuated response was characterized by decreased numbers of tumor-infiltrating CD8+ T cells (**NEW Figure 3l**) and decreased

percentage of pTBK1+ and pIRF3+ tumor cells (**NEW Figure 3m**). Additional *in vivo* data confirms that anti-tumor effects of teniposide or paclitaxel depend upon host IFNAR signaling (**NEW Supplementary Figure 2i**). Supporting *in vitro* experiments show that stimulation of B16F10 with either paclitaxel or teniposide drives co-localization of cGAS and γH2AX (**NEW Supplementary Figure 2g**), and induces *IFNβ* and *IL-6* mRNA expression in a STING or DAPK3-dependent manner (**NEW Figure 3b, 3c**). Phosphorylation of IRF3 or p65 was also significantly decreased in DAPK3-depleted B16F10 (**NEW Figure 3d, 3e**).

Together, new *in vivo* and *in vitro* data directly demonstrate that tumor-intrinsic DAPK3 expression is critical for optimal effectiveness of immunotherapy regimens that drive STING-IFNβ pathway activation in tumors, which includes partial response to 3'-3'-cGAMP and complete response to teniposide or paclitaxel. This is clearly articulated in the results section of the revised manuscript.

4) The authors claim the paracrine of type I IFNs from tumor cells to host cells elicits antitumor immunity and leads to tumor regression. They should examine immune cell infiltration in the tumors of wild-type and IFNAR KO host that are transplanted with DAPK3- or STING-deficient and sufficient cells.

As suggested by the Reviewer, we provide flow cytometry analysis of DAPK3-depleted MCA205 cells implanted into WT and IFNAR1-KO mice (**NEW Supplementary Figure 2c**), confirming that reduced infiltration of CD8+ T cells into DAPK3-depleted MCA205 tumors is abolished in IFNAR1-KO mice.

5) The authors try to conclude a role of DAPK3 in non-immunogenic tumors in response to ICB therapy and they used B16F10 melanoma model to justify this conclusion. However, B16 melanoma is an immunogenic tumor and ICB treatment almost completely inhibited tumor growth. The authors should try other non-immunogenic models to make such a conclusion.

We acknowledge that use of "*immunogenic*" among cancer immunology studies is inconsistent, and revised phrasing to specify "*IFNβ producing*" tumors such as MCA205 or paclitaxel or teniposide-treated B16F10. Notably, effect of multiple doses of single anti-PD-1 antibody administration in B16F10 tumors is partial (**NEW Figure 3g**), requiring co-administration of teniposide or paclitaxel to activate DAPK3-STING-IFNβ responses and drive synergistic anti-tumor effects and complete tumor regression *in vivo* (**NEW Figure 3j, 3k**).

6) How does DAPK3 affect K48-linked ubiquitination of STING? If DAPK3 controls basal ubiquitination and degradation of STING, the authors should examine endogenous association between DAPK3 and STING and their colocalization in subcellular compartments in unstimulated or dsDNA-transfected cells. STING is very stable with CHX treatment alone. Does STING become unstable in DAPK3-deficient cells with CHX treatment? If yes, is it rescued by MG132?

As suggested by the Reviewer, we provide new co-immunoprecipitation experiments demonstrating endogenous STING-DAPK3 interaction in unstimulated L929 (**NEW Figure 4d**). In contrast, STING and DAPK3 do not interact strongly in unstimulated THP1, as demonstrated by immunoprecipitation and confocal microscopy (**NEW Figure 5f, 5h**), in which basal STING protein levels are unaffected by DAPK3 depletion. However, cGAMP drives DAPK3-STING interaction in THP1 (**NEW Figure 5f**) and enhances STING-DAPK3 interaction in L929. We also performed immunoprecipitation in HEK293T stably expressing HA-STING (**NEW Supplementary Figure 5e**), confirming that DAPK3 interacts with STING C-terminal.

Regarding additional analysis of STING K48-linked ubiquitination, we respectfully assert that this is not directly relevant to the scope of this manuscript, due to the fact that expression of the kinase dead, tumor-associated DAPK3 D161A in MCA205 restores steady-state STING protein levels but does not rescue IFNβ production or control tumor growth *in vivo* (**Figure 2e, Supplementary Figure 2f**). Instead, we focused upon kinase-dependent effects of DAPK3 on STING K63-linked ubiquitination.

7) By using overexpression system, the authors claim that DAPK3 stabilizes STING independently of its kinase activity, and promotes K63-linked ubiquitination in a kinase activity-dependent manner. However, these experiments should be performed with DAPK3 KO cells reconstituted with WT or mutant DAPK3. In

addition, the authors identified several phosphorylated sites on LMO7 and TRIP12. They should make SA or SD mutations of LMO7 and TRIP12 to examine the roles of such mutants in regulation of K63-linked ubiquitination of endogenous STING in DAPK3-sufficient and deficient cells.

As iterated in response #1 above, CRISPR/Cas9 is not suitable for rigorous investigation of cellular DAPK3 phenotypes, due to the fact that disruption of DAPK3 using multiple sgRNA sequences and different delivery methods rendered primary or transformed stromal cells and immune cells inviable (M Takahashi and S Sharma, *unpublished results*). Stable THP1 cells constitutively expressing LMO7 S863A were also inviable (Takahashi and Sharma, *unpublished results*).

To address the Reviewer's concerns, we provide new data demonstrating that the S863A LMO7 mutant significantly impairs STING-K63 ubiquitination compared to wild type protein (**NEW Figure 7d**), which occurs due to impaired interaction of S863A LMO7 and STING (**NEW Figure 7c**). Accordingly, STING-K63 ubiquitination induced by wild type LMO7 was significantly impaired in DAPK3-depleted 293T cells (**NEW Supplementary Figure 7b**). Together, these data provide direct evidence that DAPK3-dependent phosphorylation of LMO7 is critical for STING K63-linked ubiquitination.

Minor comments

1 The knockdown efficiencies in various types of cells should be examined by Western blot assays throughout the study.

We added data confirming protein knockdown efficiency of each signaling molecule by western blotting in every relevant cell type in **Supplementary Figure 1g and Supplementary Figure 7h** (HUVEC); **Supplementary Figure 1i** (BMDM); **Supplementary Figure 2a** (MCA205); **Supplementary Figure 2b** (B16F10); **Figure 5a, NEW Supplementary Figure 1p, and Supplementary Figure 7c, 7g** (THP1); **Figure 4a, NEW Supplementary Figure 1q, and Supplementary Figure 4b, 4e** (L929).

2 In Fig 2d, total cell numbers of various immune cells should be shown.

We calculated total cell numbers in **Figure 2c**, **NEW Figure 3l, and NEW Supplementary Figure 2c.** Due to significant cell death during isolation and sorting of tumor cells, we were unable to calculate the number of pTBK1+ and pIRF3+ tumor cells *in vivo* (**NEW Figure 2f, NEW Figure 3m**), and have presented these values only as a percentage, which is in accordance with reported methodologies (Ding L et al., *Cell Rep.* 2018; Pantelidou C et al., *Cancer Discov.* 2019). This is clearly articulated in the text and figure legends.

3 Which IFNAR is knocked out in the IFNAR KO mice? IFNAR1 or IFNAR2?

IFNAR1-KO mice were used, which has been specified in results and method sections.

4 If DAPK3- or STING-knockdown affects proliferation of cells because of insufficient production of basal type I IFNs (Fig 2 and Fig S2), what is the effect by neutralizing type I IFNs with antibodies or complement of type I IFNs in the cultures of DAPK3- or STING-knockdown/KO cells?

Although baseline and inducible IFNβ levels are comparably inhibited in MCA205 and B16F10 depleted of DAPK3 or STING (**Figure 1m**, **n**, **Supplementary Figure 2e**), proliferation of STING-depleted cells is not impaired (**Supplementary Figure 2a, 2b**). Thus, effects of DAPK3 upon cell growth are likely independent of STING-IFNβ signaling, which is supported by reports that DAPK3 drives cytokinesis (Hosoba K et al., *Biochem Biophys Res Commun.* 2015). This is clarified in the results section.

5 In Fig 3c and 4f, the STING blot should be included in the WCL panels.

The STING blot is included in **NEW Figure 4c** and **NEW Figure 5d**.

6 In Fig 4d, images with higher magnification should be shown.

Higher magnification images have been provided in **NEW Figure 5h and NEW Supplementary Figure 5c**.

Decision Letter, second revision:

Subject: Decision on Nature Immunology submission NI-A29718B

Message: 15th Dec 2020

Dear Sonia,

Thank you for providing your response to the remaining concerns posed by referee Q3 on your manuscript entitled "The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING-IFNβ pathway". We are interested in the possibility of publishing your study in Nature Immunology.

As noted previously, we are overruling point 1 requesting the generation of a cKO for DAPK3 and testing this model in vivo - as that is a big ask under the current global COVID-19 conditions. However, please include the clarifications regarding the differences between the human and mouse STING proteins and their ubiquitinylation requirements, provide higher resolution confocal images of STING trafficking, and enumerating the number of tumor-infiltrating CD103+CD8a+DC in tumors implanted into IFNAR KO mice.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in this letter.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

Reporting summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots

presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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We hope to receive your revised manuscript within two weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Kind regards & "Hopeful Holidays"

Laurie

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Referee expertise:

Referee #1: Innate immunity

Referee #2: Innate immune sensor signaling

Referee #3: Innate immune sensor signaling

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The revised manuscript is considerably strengthened with several new figures which further support the model proposed. The authors have provide clearer and stronger evidence supporting the role of DAPK3 in modulating the STING response. In addition the authors have refined their discussion of the results to better explain the impact of the findings.

Reviewer #2:
Remarks to the Author:
The reviewer appreciates the large efforts the authors have undertaken to address some pertinent concerns. All in all the manuscript improved through further experimentation. The authors provide solid evidence that DAPK3 affects STING signalling, but also other innate immune signalling pathways. Their final mechanistic model remains a bit blurry, likely reflecting the complex interrelationship between the general role of ubiquitin in STING activation and cell-type specific differences as well as redundancy in between certain pathway components.

Reviewer #3:
Remarks to the Author:
In the revised manuscript, the authors performed additional experiments to justify that DAPK3 is essential for STING stabilization in unstimulated tumor cell lines and for STING activation in 2',3' cGAMP-stimulated THP-1 cell line. Although conventional knockout of DAPK3 is embryonic lethal, it is worthy to generate and examine conditional DAPK3 KO mice. Without in vivo conditional DAPK3 KO mice or cells, the conclusions are not convincing. For example, TRIM56 has been reported to catalyze K63-linked ubiquitination of STING, while studies with TRIM56 KO mice suggest that TRIM56 catalyzes mono-ubiquitination of cGAS rather than ubiquitination of STING.

Secondly, although DAPK3 is required for cGAMP-induced K63-linked ubiquitination of STING, it is dispensable for the translocation from ER to Golgi or ERGIC, which contradicts with the results from a number of studies that K63-linked ubiquitination of STING is a prerequisite for its translocation from ER to ERGIC. The quality of the confocal images is too low to support the conclusion. In addition, the levels of STING in shDAPK3 cells are almost equal to those in shControl cells in the confocal images, which is inconsistent with

the Western blotting results.

Thirdly, the authors demonstrate that deficiency of IFNAR1 in host results in hyper growth of tumor cells. They should include the percentages and numbers of various immune cells infiltrated in the tumor.

Finally, as commented above, TRIM56 is not an E3 for STING which should be deleted in Fig 8.

Author Rebuttal, third revision:

Response to Reviewer #3, Takahashi et al.

We thank the Editor and Reviewers for evaluating the revised manuscript by Takahashi et al. Here we provide a point-by-point response addressing remaining concerns of Reviewer 3. Specifically, we further revised the manuscript to provide: 1) text clarifications outlining the direct role of human TRIM56 in catalyzing K63-linked ubiquitination of human STING, and emphasizing independent regulation, by different K63-linked ubiquitination E3 ligase proteins, of STING ER-Golgi trafficking and STING-TBK interaction; 2) high-resolution, publication-quality confocal images of STING trafficking (**NEW Supplementary Figure 5a-b**), as well as all for fluorescence (**NEW Supplementary Figure 1b, 1e**) or confocal imaging (**NEW Figure 5h, NEW Supplementary Figure 2d, 2g, NEW Supplementary Figure 5a, 5c**) and western blot figures (**NEW Figure 3d, 3e, NEW Figure 4a-4e, NEW Figure 5a-5g, NEW Figure 6e, NEW Figure 7a-7d, 7f, 7g, NEW Supplementary Figure 1g, 1i, 1p, 1q, NEW Supplementary Figure 2a, 2b, 2f, NEW Supplementary Figure 4b, 4e-4h, 4k, 4l, NEW Supplementary Figure 5e, NEW Supplementary Figure 6c, 6d, 6f-6h, NEW Supplementary Figure 7a-7h**); 3) new experimental data demonstrating reduced numbers of tumor-infiltrating CD103+CD8a+DCs in IFNAR1 KO mice (**NEW Supplementary Figure 2c**), to accompany prior results for tumor-infiltrating CD8+ T cells. We also removed TRIM56 from **NEW Figure 8**, as suggested by the Reviewer. We trust that the revised manuscript is suitably rigorous and novel for publication in *Nature Immunology*. The corresponding changes in the revised manuscript are marked in blue font.

1) TRIM56 has been reported to catalyze K63-linked ubiquitination of STING, while studies with TRIM56 KO mice suggest that TRIM56 catalyzes mono-ubiquitination of cGAS rather than ubiquitination of STING.

To iterate the role of TRIM56 in catalyzing K63-linked ubiquitination of STING based upon prior studies, this was first demonstrated in STING ubiquitination assays using human TRIM56 and human STING (Tsuchida et al., *Immunity* 2010), but was not subsequently recapitulated using murine Trim56 (Wang et al., *Immunity* 2014). Notably, our experiments in 293T cells over-expressing human TRIM56 and human STING are consistent with the published methodology and results of Tsuchida et al. Importantly, these results neither rule out nor contradict a role for TRIM56 in catalyzing mono-ubiquitination of cGAS, which was reported by Seo et al., *Nature Communications* 2018. In the revised manuscript, we have specified in the Results and Discussion sections that experimental results relating to K63-linked ubiquitination of STING by TRIM56 specifically pertain to the human protein, and cite the manuscript by Tsuchida et al. Also, to avoid confusion regarding species-specific differences in TRIM56 function in the STING pathway, we removed TRIM56 from the model in **NEW Figure 8**, as suggested by the Reviewer.

2) Although DAPK3 is required for cGAMP-induced K63-linked ubiquitination of STING, it is dispensable for the translocation from ER to Golgi or ERGIC, which contradicts with the results from a number of studies that K63-linked ubiquitination of STING is a prerequisite for its translocation from ER to ERGIC.

To clarify the pleiotropic role of ubiquitination in regulating STING activation, numerous published reports show that agonist-induced K63-linked ubiquitination of human STING drives trafficking from ER to Golgi (Ni et al., *Sci Immunol* 2017) and interaction with TBK1 (Tsuchida T et al., *Immunity* 2010; Zhang et al., *JBC* 2012; Ni et al., *Sci Immunol* 2017). Notably, these studies clearly indicate that for human STING, trafficking and TBK1 interaction are distinct events subject to independent regulation by different K63-linked ubiquitin E3 ligases (Tsuchida T et al., *Immunity* 2010; Ni et al., *Sci Immunol* 2017). For example, Tsuchida et al showed that K63-linked ubiquitination of human STING by human TRIM56 is specifically required for STING-TBK1 interaction, but dispensable for STING trafficking. Furthermore, Ni et al showed that K63-linked ubiquitination of human STING by human MUL1 is specifically required for STING trafficking, which indirectly affects STING-TBK1 interaction. Thus, our results are consistent with published literature which indicates that K63-linked ubiquitination of human STING can exert distinct regulatory effects upon trafficking or interaction with TBK1. This has been clarified in the Results and Discussions sections of the revised manuscript.

The quality of the confocal images is too low to support the conclusion.

To increase image quality, we provide high-resolution, publication quality versions of these confocal images (**NEW Supplementary Figure 5a-b**), as well as all fluorescence images (**NEW Supplementary Figure 1b, 1e**) or confocal images imaging (**NEW Figure 5h, NEW Supplementary Figure 2d, 2g, NEW Supplementary Figure 5a, 5c**) and western blot data (**NEW Figure 3d, 3e, NEW Figure 4a-4e, NEW**

Figure 5a-5g, NEW Figure 6e, NEW Figure 7a-7d, 7f, 7g, NEW Supplementary Figure 1g, 1i, 1p, 1q, NEW Supplementary Figure 2a, 2b, 2f, NEW Supplementary Figure 4b, 4e-4h, 4k, 4l, NEW Supplementary Figure 5e, NEW Supplementary Figure 6c, 6d, 6f-6h, NEW Supplementary Figure 7a-7h).

3) The levels of STING in shDAPK3 cells are almost equal to those in shControl cells in the confocal images, which is inconsistent with the Western blotting results.

To clarify this important point for the Reviewer, THP1 cells were used in confocal imaging studies. Notably, loss of DAPK3 in THP1 cells does not affect basal STING protein levels, as demonstrated by western blotting (**Figure 5a**). Thus, confocal images are consistent with equal levels of STING protein in control and DAKP3-depleted THP1 cells. This is stated in the Results section of the revised manuscript.

4) The authors demonstrate that deficiency of IFNAR1 in host results in hyper growth of tumor cells. They should include the percentages and numbers of various immune cells infiltrated in the tumor.

We previously included data showing reduced numbers of tumor-infiltrating CD8+ T cells in IFNAR1 knockout mice (**Supplementary Figure 2c**). As suggested by the Reviewer, we added measurements of CD103+CD8a+DC numbers in **NEW Supplementary Figure 2c**.

5) Finally, as commented above, TRIM56 is not an E3 for STING which should be deleted in Fig 8.

As the Reviewer suggested, to avoid any confusion between differences in human TRIM56 and mouse Trim56, we removed TRIM56 from **NEW Figure 8**.

Decision Letter, third revision:

Subject: Nature Immunology - NI-A29718C pre-edit

Message: Our ref: NI-A29718C

17th Jan 2021

Dear Sonia,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "The tumor suppressor kinase DAPK3 drives tumor-intrinsic immunity through the STING-IFNβ pathway" (NI-A29718C).

I am attaching the edited manuscript. The manuscript is generally well-written, I have only some minor comments, mostly dealing with journal style.

I have made changes marked in tracked-changes, queries in red and comments are embedded throughout the manuscript, so please have the view comments option enabled. Also, the manuscript was reviewed by internal reproducibility checkers and I have appended their comments in the attachments on the reproducibility forms that you provided upon submission.

Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the points below. We won't be able to proceed further without this detailed response.

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Figures and Tables:

All figures and tables, including Extended Data, must be cited in the text in numerical order.

Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

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Graph axes should start at zero and not be altered in scale to exaggerate effects. A 'broken' graph can be used if absolutely necessary due to sizing constraints, but the break must be visually evident and should not impinge on any data points.

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