

TRIM23 mediates cGAS-induced autophagy in anti-HSV defense

Corresponding Author: Dr Michaela Gack

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript reports a key role for TRIM23 in cGAS/STING-mediated autophagy. The authors have previously identified TRIM23 as playing important roles in virus-induced autophagy. Here, they carried out experiments in human cell lines, patient-derived cell lines, and in knockout mice to test the mechanism of TRIM23 action in autophagy and the TRIM23-autophagy axis' relevance to antiviral defense. Their current model involves cGAS/STING promoting TBK1 activation, with TBK1 subsequently activating TRIM23's auto-ubiquitination and GTPase activity by phosphorylation of the TRIM23 RING domain; leading to autophagy activation.

Overall, the manuscript represents an important advance in the understanding of how TRIM23 orchestrates antiviral autophagy. TRIM23 is a member of the TRIM super-family of ubiquitin ligases, many of which also have roles in autophagy. With additional studies, the authors principal findings could potentially be expanded to better understand the mechanisms underlying how other TRIMs regulate autophagy following other physiological stimuli.

Nevertheless, there are several weaknesses that should be addressed prior to publication in Nature Communications. Most importantly, the authors need to provide more evidence that virus-induced autophagy is reduced in TRIM23 knockout mice and should better define the apparent positive feed-back loop between TBK1 and TRIM23. Additionally, as detailed below, there were some instances in which the rigor of statistical analysis should be improved.

Major comments:

Figure 1E, F: While I understand the technical difficulties of measuring autophagy in mouse tissues, one experiment measuring p62 protein abundance in tissue is insufficient to support the authors' key point regarding reduced virus-induced autophagy in mouse brains following HSV-1 infection. Since there is no one good way to do this, the authors should provide multiple orthogonal assays that together could provide some picture of autophagy activation and flux. These assays should also include samples from mock-infected WT and TRIM23 knockout mice. Since p62 protein level can be determined by many factors in addition to autophagic degradation, relying on p62 alone is not convincing.

Figure 1H, J, L and 3D: The p62 data in these panels could be strengthened by demonstrating that the decrease in p62 levels following infection of siControl cells was autophagy-dependent, which could be done using a lysosomal inhibitor such as bafilomycin A1. This is standard practice also when LC3-II conversion is measured to ensure that effects are autophagy induction rather than changes in flux.

One opportunity to broaden the scope of this study would be to determine if other TRIMs' RING domains have similar serine residues to TRIM23 S39 that could be phosphorylated to activate E3 ligase activity. Do other TRIMs have a putative TBK1 phosphosite at roughly the same position in their RING domains, or is TRIM23 unique in this respect?

In a previous study, the authors reported that TRIM23 was required for TBK1 activation in virus-induced autophagy. On the other hand, the current study places TBK1 up-stream of TRIM23. The most likely explanation for these seemingly discordant results is that there is a feed-forward cycle involving the two proteins. The authors should undertake experiments to more clearly delineate how this cycle works. How do TRIM23-TBK1 complexes assemble in response to virus infection and/or cGAS activation? Does the assembly require TRIM23 ubiquitin ligase activity? Is TBK1 ubiquitinated by TRIM23? How does this impact TBK1 activation?

Figure 4B: The authors test whether over-expression of myc-PINK1 can induce TBK1 phosphorylation. In the absence of mitochondrial depolarization, this experiment is unlikely to yield a positive result since PINK1 is typically cleaved and inactivated following import into the mitochondria. As such, it may be best to remove this data set.

Please explain why the attenuated mutHSV-1 appeared to be more virulent in Figure 1A than the WT virus in Figure S1B.

The GTPase experiment in Figure 3A should be repeated with ARF-deleted TRIM23 as a negative control.

Quantitation of colocalization or of relative protein levels from immunoblots should be provided from 3 independent experiments. In particular, please provide quantitation for Figure 3F, Figure 4A, Figure 5A, and 6F.

Minor comments:

Please specify that Calyculin A is a phosphatase inhibitor.

Figure 3F: It appears that TRIM23 S39A loses the ability to form puncta in cells. If this is accurate, perhaps the authors could mention this in the text.

Figure 5B: please label which band is cGAS and which band is STING.

Figure 5: Please explain why Viperin is used as a control in these experiments.

Please write out the full term for HSE.

Line 219-221: The authors say that “bioinformatics” was used to identify candidate kinases, but I could find no information on what was specifically done. Please provide information about the websites/software that was used for this analysis.

Please check the figure legends for Figures 3, 5, and S1; which had some discrepancies between what was in the legend versus what was in the corresponding figure.

Reviewer #2

(Remarks to the Author)

In this study by Acharya et al., the authors report a molecular mechanism by which TRIM23 executes cGAS-mediated antiviral autophagy. The study extends their previous findings, in which they identified TRIM23, using a high throughput screen of TRIM proteins, as an activator of HSV-1-induced autophagy. The current study explored the molecular determinants of TRIM23-mediated autophagy activation in HSV-1-infected cells, mice, and patients. Through genetic ablation studies and using fibroblasts from HCE patients, the authors found that the upstream kinase, TBK1, was the key player in activating autophagy. TBK1 was responsible for phosphorylating TRIM23 at Serine 39, resulting in its autoubiquitination and GTPase activity, leading to autophagy. The study is well-rounded in that it starts with observation in a mouse model, continues with in-depth mechanistic studies in vitro, and finally validates the phenomena and mechanism in patient samples, highlighting the biological and translational significance of their findings. Although the study, as presented currently, is solid, with appropriate rigors, some additional points, noted below, should be considered to strengthen the manuscript further:

Major points:

1. All mechanistic studies have been performed using mutHSV1, which doesn't inhibit autophagy, as a strong activator of the autophagy pathway. However, the lack of ICP34.5 in mutHSV-1 also makes it a potent activator of interferon responses, particularly, as shown earlier, IRF3 activation, and probably other yet unidentified pathways. Whether such responses affect autophagy activation by TRIM23 may not be ruled out. The authors can use the wild-type viral strain to validate some of their molecular mechanisms. This is particularly relevant since animal and human studies were conducted using the wild-type viral strain.
2. The study relies heavily on ectopic (and over) expression systems. Some of their mechanistic studies, inducing the phosphorylation/ubiquitination of TRIM23, should be analyzed using endogenous protein. For example, is TRIM23 phosphorylated in mouse and human studies? Since all the tools are in place, this could add significantly to the conclusion.
3. In their previous study, the authors reported that TRIM23 activates TBK1, causing its phosphorylation. The current study shows the reciprocal regulation. How do they reconcile these molecular effects?
4. Since TRIM23 mediates antiviral activity in IFNAR1 KO cells, is this due to autophagy activation? It will be insightful to compare the TRIM23/TBK1-mediated autophagy induction in Wt and IFNAR1 KO cells.
5. It will be interesting to examine the viral replication in patient fibroblasts with the constitutively active TRIM23 mutant.

Minor points:

1. Please show the TRIM23 protein level for knockout and knockdown confirmation.
2. Fig 3: the panels are incorrectly labeled and do not match the legends.

Reviewer #3

(Remarks to the Author)

Acharya and colleagues investigated the role of TRIM23 in cGAS-dependent antiviral autophagy. They report that the cGAS-STING-mediated classical autophagic pathway, in the context of HSV infection and dsDNA stimulation, requires TBK1-

triggered TRIM23 S39 phosphorylation and subsequent autoubiquitination. The study addresses an interesting topic; however, the overall innovation of the research is limited, and the data presented are less compelling. The analysis appears somewhat cursory, and the interpretation of the data lacks thorough consideration. Thus, there are some important and interesting questions raised from this work that the manuscript does not address and would clearly increase the significance of the study.

Major comments

1. A hallmark of autophagic flux is the degradation of the selective autophagy receptor p62 and the conversion of LC3B-I to LC3B-II. The study appears somewhat inconsistent in its approach, lacking a unified standard. For instance, the evaluation of autophagic flux varies between p62 degradation, p62 phosphorylation, LC3I-II conversion via immunoblotting, and GFP-LC3B puncta formation. For example, in Figures 1E, 1J, and 1L, additional markers, such as LC3I-II conversion, are needed to better demonstrate that TRIM23 is critical for autophagic induction, beyond just p62 protein levels as shown in Figure 1H. Furthermore, there is inconsistency in the plasmid constructs used, alternating between myc-TRIM23 and flag-TRIM23.
2. Figure 4 relies heavily on overexpression systems, particularly in 293T cells. Given that these cells endogenously express TRIM23, evaluating the effects of endogenous TRIM23 expression is more convincing.
3. Previously, Sparrer, K.M.J. et al. from the same research group demonstrated that TRIM23 mediates virus-induced autophagy through the activation of TBK1 (Nature Microbiology 2, 1543-1557 (2017)). In this study, they showed that TBK1 is the kinase responsible for phosphorylating and activating TRIM23, which is unsurprising. TRIM23 plays a pivotal role in the autophagic process triggered by various viruses, including HSV, EMCV, IAV, and SINV. In the context of HSV infection, it is logical that autophagy is triggered via cGAS. I am quite interested in exploring the function of the TBK1-TRIM23 axis in autophagy during IAV or SINV infection. Whether this process involves MAVS or TRIF is indeed an intriguing question. If the TBK1-TRIM23 axis is found to be a key regulator of autophagy upon activation by various upstream signaling pathways, it would significantly enhance the importance of this study.
4. It is intriguing that TRIM23 specifically mediates autophagy in the cGAS-STING pathway, but not in the RLR or TLR pathways. Given that key adaptors in the DNA sensing, RLR, and TLR pathways—namely STING, MAVS, and TRIF—share a conserved motif that interacts with TBK1 and requires TBK1 phosphorylation to activate downstream signaling, could you provide a detailed explanation for why the TBK1-TRIM23 axis is a critical regulator of autophagy specifically in the cGAS pathway? What are the potential mechanisms underlying this specificity?
5. Additionally, could you elaborate on the nature of the binding relationship between the STING-TBK1-TRIM23 complex? Specifically, how does TBK1 interact with STING and TRIM23 within this complex, and what role does this interaction play in mediating cGAS-STING-induced autophagy? Understanding the structural and functional dynamics of this interaction could provide deeper insights into the specificity of the TBK1-TRIM23 axis in STING-triggered autophagy regulation.
6. The patient samples with HSE provide strong evidence for the correlation between TBK1 and TRIM23. I am curious whether functional mutations in TRIM23 are present in HSE patients. Identifying such mutations would provide more direct evidence of TRIM23's importance in the disease context.

Minor comments

1. The current title seems out the scope of the study. To ensure accuracy, I suggest revising the title to "TRIM23 Mediates cGAS-Induced Autophagy in Anti-HSV Defense".
2. More referenced is needed in line 50-52 :cGAS-STING activation induces "classical" p62/SQSTM1-mediated autophagy as well as "non-canonical" autophagy or CASM (conjugation of ATG8 to single membranes)10, 11, 12, 13, 14. (PMID: 36872914, PMID: 19926846, PMID: 34453126, PMID: 37535724)
3. In reviewing Figures 1C, 1G, and 1K, it is not clear on what basis the specific viral proteins were chosen to indicate viral replication. The selection criteria for these viral proteins should be clearly stated, as the current presentation appears somewhat arbitrary. Additionally, Figure 1H shows a test for viral titer instead of RT-qPCR.
4. In Supplementary Figures S2A-C, the selection of representative ISGs appears limited. Including a broader range of ISGs or providing transcriptomic data could offer a more comprehensive view of the ISG response. This additional information would strengthen the interpretation of the data and provide a more thorough understanding of the gene expression changes associated with the experimental conditions.
5. The figure legends (3d-f) need to be reordered for clarity.
6. In Figure 3H, in addition to presenting the viral titer, it would be beneficial to include data on HSV-specific antibodies to demonstrate the viral potency.
7. In Figure 4A, a negative control is missing to demonstrate the specificity of the phosphorylated bands.
8. In Figures 3C, 4J, and 5D, the statistical methods used are inconsistent, which makes the analysis appear arbitrary.
9. In all the virus infection experiments, including those in Western blot analyses, there is a lack of data on HSV-related protein expression to confirm the consistency of the viral infection, as seen in Figure 5A.
10. In Figure 5A, the expression levels of LC3I/II need to be provided.
11. In Figure 4B, several key details are missing: there is no reference to internal controls in the whole cell lysate (WCL), nor are the expressions of Flag and phosphorylated proteins provided. Additionally, the expression of HA and Myc in the immunoprecipitation (IP) experiments is not shown. It is also unclear whether TBK1 interacts with TRIM23. Similar issues are noted in Figures 4C, 4F-H, 5B and 6B.
12. Figure 4D , 4F lacks a negative control.
13. Usually, p62 phosphorylation correlates with changes in p62 levels. However, this correlation does not appear to hold in Figures 5G and 5H. Please clarify
14. Please provide LC3 expression in Figure 5G.
15. Typically, the level of autophagy is assessed by comparing the ratio of LC3I to LC3II. However, in Figure 5H, the transition between LC3I and LC3II does not support the conclusion presented.
16. CCL5 data is missing in S1C.
17. S4B LC3I/LC3II ratio is quite different from 5H.
18. Where is the LC3I band in S4G?

19. TBK1-p should be provided in figure 6A.

20. Rephrase lines 127-129, as A549 cells have not shown data on p62 degradation. The text description does not match the data, and the conclusion appears hasty and lacking in rigor.

21. Please provide references for the GFP-LC3B puncta assay.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the revised manuscript, Acharya et al. addressed most of the reviewers' comments and questions and they improved their figures significantly. The co-reviewer and I are both in support of this manuscript's acceptance to Nature Communications. The following minor comments should be addressed to improve the final manuscript.

Minor Comments:

- There is no statistical information presented in the graphs of Supplementary Figures S1f–i, S2b–e, S4a–b, S5a and S5k, despite the figure legend stating that statistical tests were conducted. Please clarify this and indicate whether only significant values are shown, or provide the relevant statistical details.
- Line 164: I think the authors mean “dispensable” instead of “indispensable”.
- While not required, a diagram displaying the authors' model for the STING-TBK1-TRIM23 feed-forward loop would be helpful.

Reviewer #2

(Remarks to the Author)

The authors have addressed the previous concerns satisfactorily using new expts.

Reviewer #3

(Remarks to the Author)

I appreciate the authors' efforts in addressing my previous comments. The revisions have significantly improved the manuscript, and I am satisfied with the responses provided.

To enhance the clarity and impact of your manuscript, I strongly recommend including a model figure that summarizes the key findings of your study and their implications for the cGAS-STING-autophagy axis. This figure should visually depict the proposed molecular mechanisms, including the phosphorylation of TRIM23 by TBK1, the formation of the STING-TBK1-TRIM23 complex, and the subsequent activation of autophagy. The model should highlight the novel aspects of your work, such as the unique role of TRIM23 S39 phosphorylation and the feed-forward loop between TBK1 and TRIM23.

Additionally, the figure could include a discussion of the broader implications of your findings, such as potential therapeutic targets or connections to autophagy-related diseases. Please ensure the figure is visually clear and accompanied by a concise legend to aid reader comprehension.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Point-by-point response to reviewer's comments

We would like to thank the reviewers for their constructive feedback and very helpful suggestions. We have addressed the points raised by the reviewers by performing a series of additional experiments, which led to a total of 51 new or improved subpanels in the revised manuscript. Specifically, we performed experiments to further strengthen (1) TRIM23 S39 phosphorylation at the endogenous protein level in human and mouse cells; (2) endogenous TRIM23 S39 phosphorylation and TRIM23-TBK1 interaction in the context of WT HSV-1 (in addition to mutHSV-1) infection; (3) a proposed 'feed-forward' circuit of TBK1 and TRIM23 for autophagy activation in anti-HSV-1 defense; and (4) endogenous TRIM23-TBK1-STING complex formation upon HSV-1 infection or dsDNA stimulation. Additionally, we have improved several data sets according to the reviewers' suggestions and clarified or discussed several points raised. We believe that these additional data and clarifications greatly improved our manuscript.

Reviewer #1 (TRIM family member, innate immunity) (Remarks to the Author):

This manuscript reports a key role for TRIM23 in cGAS/STING-mediated autophagy. The authors have previously identified TRIM23 as playing important roles in virus-induced autophagy. Here, they carried out experiments in human cell lines, patient-derived cell lines, and in knockout mice to test the mechanism of TRIM23 action in autophagy and the TRIM23-autophagy axis's relevance to antiviral defense. Their current model involves cGAS/STING promoting TBK1 activation, with TBK1 subsequently activating TRIM23's auto-ubiquitination and GTPase activity by phosphorylation of the TRIM23 RING domain, leading to autophagy activation.

Overall, the manuscript represents an important advance in the understanding of how TRIM23 orchestrates antiviral autophagy. TRIM23 is a member of the TRIM super-family of ubiquitin ligases, many of which also have roles in autophagy. With additional studies, the authors principal findings could potentially be expanded to better understand the mechanisms underlying how other TRIMs regulate autophagy following other physiological stimuli.

→ **Reply:** We thank the reviewer for this very positive evaluation stating that our study represents an important conceptual advancement to the field.

Nevertheless, there are several weaknesses that should be addressed prior to publication in Nature Communications. Most importantly, the authors need to provide more evidence that virus-induced autophagy is reduced in TRIM23 knockout mice and should better define the apparent positive feed-back loop between TBK1 and TRIM23. Additionally, as detailed below, there were some instances in which the rigor of statistical analysis should be improved.

→ **Reply:** We have now performed several additional experiments to comprehensively address the points raised by the reviewer.

Major comments:

1. Figure 1E, F: While I understand the technical difficulties of measuring autophagy in mouse tissues, one experiment measuring p62 protein abundance in tissue is insufficient to support the

authors' key point regarding reduced virus-induced autophagy in mouse brains following HSV-1 infection. Since there is no one good way to do this, the authors should provide multiple orthogonal assays that together could provide some picture of autophagy activation and flux. These assays should also include samples from mock-infected WT and TRIM23 knockout mice. Since p62 protein level can be determined by many factors in addition to autophagic degradation, relying on p62 alone is not convincing.

→ **Reply:** We thank the reviewer for raising these important points. As requested, we have now analyzed autophagy in mouse brains more rigorously by including samples from mock infected animals using western blot and immunohistochemistry analyses (with additional data on HSV-1 antigen staining from sequential brain sections) (**Fig. 1e and Supplementary Fig. 1c**). We also show the HSV-1 antigen expression more comprehensively in multiple anatomical locations of the brain (**Fig. 1d**). As noted by the reviewer, we faced technical difficulty in measuring autophagy by other readouts in mouse brain tissues. To still address the reviewer's point, we now show in multiple physiologically relevant cell types the crucial role of TRIM23 in HSV-1-induced autophagy by other readouts (e.g., LC3-I-to-II conversion) in addition to measuring p62 abundance (**Fig. 1g, i, k**).

2. Figure 1H, J, L and 3D: The p62 data in these panels could be strengthened by demonstrating that the decrease in p62 levels following infection of siControl cells was autophagy-dependent, which could be done using a lysosomal inhibitor such as bafilomycin A1. This is standard practice also when LC3-II conversion is measured to ensure that effects are autophagy induction rather than changes in flux.

→ **Reply:** As suggested by the reviewer, we have now performed experiments using bafilomycin A1 in multiple cell types (i.e., human and mouse neurons, human fibroblasts) to demonstrate that the decrease in p62 levels following HSV-1 infection depends on autophagy, but not proteasomal degradation (**Supplementary Fig. 1e**). Of note, these data are consistent with published reports (e.g., Gobeil and Leib 2012 [PMID: 23073763], Waisner and Kalamvoki 2019 [PMID: 31375597], and Bjørkøy et al., 2009 [PMID: 19200883]).

3. One opportunity to broaden the scope of this study would be to determine if other TRIMs' RING domains have similar serine residues to TRIM23 S39 that could be phosphorylated to activate E3 ligase activity. Do other TRIMs have a putative TBK1 phospho-site at roughly the same position in their RING domains or is TRIM23 unique in this respect?

→ **Reply:** We thank the reviewer for this insightful comment. We have now performed sequence alignments of the RING domain of all human TRIMs and interestingly found that 13 TRIMs have a serine or threonine in their RING domain at the corresponding site as S39 in TRIM23. However, based on our preliminary analysis of kinase consensus motives, these sites don't appear to be TBK1 sites, rather consensus sites for other kinases. We agree with the reviewer that this may indicate that the RING E3 ligase activity of these TRIM proteins is regulated by kinase-dependent phosphorylation. However, since this would require careful analysis of how phosphorylation affects each TRIM's E3 activity and biological function, we think these studies are outside the scope of our current manuscript. We have added the following sentence to our discussion "To our knowledge, this is the first phosphorylation-dependent 'on/off' switch of a TRIM E3 ligase. Of note, some TRIM proteins harbor in their RING domain a Ser/Thr residue at the site equivalent to TRIM23 S39, providing a mandate for exploring whether similar regulatory mechanisms exist for other TRIM proteins or more broadly other RING E3 ligases (page 25-26;

Line 545-550)".

4. In a previous study, the authors reported that TRIM23 was required for TBK1 activation in virus-induced autophagy. On the other hand, the current study places TBK1 up-stream of TRIM23. The most likely explanation for these seemingly discordant results is that there is a feed-forward cycle involving the two proteins. The authors should undertake experiments to more clearly delineate how this cycle works. How do TRIM23-TBK1 complexes assemble in response to virus infection and/or cGAS activation? Does the assembly require TRIM23 ubiquitin ligase activity? Is TBK1 ubiquitinated by TRIM23? How does this impact TBK1 activation?

→ **Reply:** We have now performed a comprehensive set of experiments to dissect the molecular details of this feed-forward cycle of the TRIM23-TBK1 axis in autophagy, which led to one additional main figure and one additional supplementary figure (New **Fig. 6 and Supplementary Fig. 6** of the revised manuscript). These new data show that TRIM23 C34A and the GTPase-dead mutant K458I were effectively phosphorylated by TBK1 (New **Supplementary Fig. 6b**), while TRIM23 Δ ARF (TBK1-binding-deficient) and TRIM23 Δ RING (lacking S39) were not, suggesting that TBK1 acts as upstream kinase for TRIM23 leading to its activation. After HSV-1 infection or dsDNA-induced cGAS-STING activation, STING, TBK1 and TRIM23 form a complex which promotes the phosphorylation of TRIM23 at S39 by TBK1 at the Golgi apparatus (New **Fig. 6a, b; Supplementary Fig. 6c, d**); this phosphorylation event then triggers TRIM23 auto-ubiquitination of the ARF domain promoting GTP-GDP cycling; GTPase activity then facilitates higher-order assembly of bound TBK1 as well as trafficking of the activated TRIM23-TBK1 complex to autophagosomes, ultimately leading to p62 phosphorylation by TBK1 (Sparrer et al., 2017, PMID: 28871090). This new comprehensive set of data, together with our previously published study (Sparrer et al., 2017, PMID: 28871090), suggest an intricate feed-forward activation loop that regulates TBK1-TRIM23-induced autophagy. We have also discussed in detail this proposed feed-forward circuit in the Discussion section (page 26, lines 550-556). Regarding the reviewer's point about whether TBK1 is ubiquitinated by TRIM23, we have published this in our previous publication (Sparrer et al., 2017; Suppl. Fig. 8c, d), which suggested that the TRIM23-induced ubiquitination of TBK1 likely occurs late during infection when TRIM23 helps TBK1 to phosphorylate p62 at autophagosomes, although the detailed function of this PTM event warrants further investigation and whether it is a direct ubiquitination event by TRIM23 (as TBK1 is ubiquitinated by several other E3 ligases). Nevertheless, our new data clearly showed that TRIM23 C34A mutant effectively binds to and is phosphorylated by TBK1 (New **Supplementary Fig. 6a, b**), which demonstrates that TRIM23's E3 ligase activity is dispensable for its initial activation by TBK1.

5. Figure 4B: The authors test whether over-expression of myc-PINK1 can induce TBK1 phosphorylation. In the absence of mitochondrial depolarization, this experiment is unlikely to yield a positive result since PINK1 is typically cleaved and inactivated following import into the mitochondria. As such, it may be best to remove this data set.

→ **Reply:** We thank the reviewer for pointing this out. We have now removed the PINK1 data as per the reviewer's suggestion.

6. Please explain why the attenuated mutHSV-1 appeared to be more virulent in Figure 1A than the WT virus in Figure S1B.

→ **Reply:** We observed ~20% mortality in WT mice infected intracranially with 5×10^5 PFU of mutHSV-1. A similar dose of the WT virus is highly lethal in mice, which would not allow us to see differences in survival between WT and *Trim23*^{-/-} mice. We therefore used a lower dose for WT virus (1×10^4 PFU), which we have described in the respective figure legends (**Fig. 1a; Supplementary Fig. 1b**)

7. The GTPase experiment in Figure 3A should be repeated with ARF-deleted TRIM23 as a negative control.

→ **Reply:** We have now repeated this experiment and included the TRIM23-ΔARF (GTPase ‘null’ mutant) as a negative control (New **Fig. 3a**).

8. Quantitation of colocalization or of relative protein levels from immunoblots should be provided from 3 independent experiments. In particular, please provide quantitation for Figure 3F, Figure 4A, Figure 5A, and 6F.

→ **Reply:** As suggested by the reviewer, we have now included quantification for these data (New **Fig. 3f, 7g; New Supplementary Fig. 4b, 5b**).

Minor comments:

1. Please specify that Calyculin A is a phosphatase inhibitor.

→ **Reply:** We have added this information to the respective figure legend (**Fig. 2d**).

2. Figure 3F: It appears that TRIM23 S39A loses the ability to form puncta in cells. If this is accurate, perhaps the authors could mention this in the text.

→ **Reply:** This is correct. As suggested, we have now described this observation in the main text (page 11, line 225-227).

3. Figure 5B: please label which band is cGAS and which band is STING.

→ **Reply:** We have now added the labeling for these proteins to the blot (**Fig. 5d** of the revised manuscript).

4. Figure 5: Please explain why Viperin is used as a control in these experiments.

→ **Reply:** We have included Viperin (a representative ISG) as a control to ensure that poly(dG:dC) treatment effectively worked under these experimental conditions, and have now briefly explained this in the legend (**Fig. 5h**).

5. Please write out the full term for HSE.

→ **Reply:** We have now described this terminology at its first appearance (page 20, line 415-416).

6. Line 219-221: The authors say that “bioinformatics” was used to identify candidate kinases, but I could find no information on what was specifically done. Please provide information about the websites/software that was used for this analysis.

→ **Reply:** We have now added this information to the Methods section (page 40, line 874-878).

7. Please check the figure legends for Figures 3, 5, and S1; which had some discrepancies between what was in the legend versus what was in the corresponding figure.

→ **Reply:** We apologize for these discrepancies. We have now corrected this.

Reviewer #2 (Autophagy) (Remarks to the Author):

In this study by Acharya et al., the authors report a molecular mechanism by which TRIM23 executes cGAS-mediated antiviral autophagy. The study extends their previous findings, in which they identified TRIM23, using a high throughput screen of TRIM proteins, as an activator of HSV-1-induced autophagy. The current study explored the molecular determinants of TRIM23-mediated autophagy activation in HSV-1-infected cells, mice, and patients. Through genetic ablation studies and using fibroblasts from HCE patients, the authors found that the upstream kinase, TBK1, was the key player in activating autophagy. TBK1 was responsible for phosphorylating TRIM23 at Serine 39, resulting in its autoubiquitination and GTPase activity, leading to autophagy.

The study is well-rounded in that it starts with observation in a mouse model, continues with in-depth mechanistic studies in vitro, and finally validates the phenomena and mechanism in patient samples, highlighting the biological and translational significance of their findings. Although the study, as presented currently, is solid, with appropriate rigors, some additional points, noted below, should be considered to strengthen the manuscript further.

→ **Reply:** We thank the reviewer for these very positive comments about our study including its biological and translational significance.

Major points:

1. All mechanistic studies have been performed using mutHSV1, which doesn't inhibit autophagy, as a strong activator of the autophagy pathway. However, the lack of ICP34.5 in mutHSV-1 also makes it a potent activator of interferon responses, particularly, as shown earlier, IRF3 activation, and probably other yet unidentified pathways. Whether such responses affect autophagy activation by TRIM23 may not be ruled out. The authors can use the wild-type viral strain to validate some of their molecular mechanisms. This is particularly relevant since animal and human studies were conducted using the wild-type viral strain.

→ **Reply:** We thank the reviewer for raising this point. As suggested, we have now validated our key molecular findings with WT HSV-1. Specifically, we have included new data that show that, similar to mutHSV-1 infection, WT virus infection effectively elicits (1) phosphorylation of endogenous TRIM23 at S39 (New **Fig. 2f, g**) and (2) colocalization of endogenous TRIM23 with activated TBK1 (New **Fig. 4h**). In our animal studies, we used both WT HSV-1 as well as mutHSV-1 to demonstrate the antiviral role of TRIM23-mediated autophagy (**Fig. 1a**, and **Supplementary Fig. 1b**). Regarding the reviewer's point about the potential influence of IFN responses on autophagy activation by TRIM23, we have now tested endogenous p62 degradation by ectopic expression of TRIM23 in cells in which IFNAR signaling was blocked by anti-IFNAR2 antibody. This showed equivalent p62 degradation triggered by TRIM23 with or

without IFNAR-signal inhibition (New **Supplementary Fig. 2f, g**). TBK1 was also included in this assay. Of note, TRIM23 is not an IFN-stimulated gene either (in contrast to many other TRIM proteins) (Sparrer et al., 2017).

2. The study relies heavily on ectopic (and over) expression systems. Some of their mechanistic studies, inducing the phosphorylation/ubiquitination of TRIM23, should be analyzed using endogenous protein. For example, is TRIM23 phosphorylated in mouse and human studies? Since all the tools are in place, this could add significantly to the conclusion.

→ **Reply:** We thank the reviewer for raising this important point. We have now performed several additional experiments to strengthen our molecular findings on TRIM23 activation at the endogenous protein level. Our new data show (1) S39 phosphorylation of endogenous TRIM23 following HSV-1 infection (New **Fig. 2f, g, h, i**) in a TBK1-dependent manner (New **Fig. 4f, g**); (2) a crucial role of endogenous cGAS-STING activation in TRIM23 S39 phosphorylation (New **Fig. 5b, c**); (3) colocalization of activated (i.e., S39-phosphorylated) TRIM23 with TBK1 at the endogenous protein level (New **Fig. 4h; Fig. 5K, l; Fig. 6a, b**); and (4) STING-signaling induced colocalization of activated endogenous TRIM23 with the Golgi at early times, while colocalization with autophagosomes at later times (New **Supplementary Fig. 6c, d**). In addition, we show that endogenous TRIM23 is phosphorylated at S39 also in mouse cells (New **Supplementary Fig. 3b, c**). We believe that these data further strengthened our findings of TRIM23 activation by direct phosphorylation at S39, and further, the overall concept of a cGAS/STING- TBK1-TRIM23 axis in autophagic defense against HSV-1 infection.

3. In their previous study, the authors reported that TRIM23 activates TBK1, causing its phosphorylation. The current study shows the reciprocal regulation. How do they reconcile these molecular effects?

→ **Reply:** We thank the reviewer for raising this point. Of note, the same point was raised also by reviewer #1. We have now performed a comprehensive set of experiments which indicate an intricate feed-forward cycle of the TRIM23-TBK1 axis in autophagy. These new data led to one additional main figure and one additional supplementary figure (New **Fig. 6 and Supplementary Fig. 6**). Specifically, our new data show that TRIM23 C34A and the GTPase-dead mutant K458I were effectively phosphorylated by TBK1 (New **Supplementary Fig. 6b**), while TRIM23 Δ ARF (TBK1-binding-deficient) and TRIM23 Δ RING (lacking S39) were not, suggesting that TBK1 acts as upstream kinase for TRIM23 leading to its activation. Upon HSV-1 infection or dsDNA-induced cGAS-STING activation, STING, TBK1 and TRIM23 form a trimolecular complex which promotes TRIM23 S39 phosphorylation by TBK1 at the Golgi apparatus (New **Fig. 6a, b; Supplementary Fig. 6c, d**); this phosphorylation event then triggers TRIM23 auto-ubiquitination of the ARF domain promoting GTP-GDP cycling; GTPase activity then facilitates higher-order assembly of bound TBK1 as well as trafficking of the activated TRIM23-TBK1 complex to autophagosomes, ultimately leading to p62 phosphorylation by TBK1 (Sparrer et al., 2017; PMID: 28871090). We have now also discussed in more detail this proposed feed-forward circuit in the Discussion section (page 26, lines 550-556). Please see also our response to reviewer #1 (major comment #4).

4. Since TRIM23 mediates antiviral activity in IFNAR1 KO cells, is this due to autophagy activation? It will be insightful to compare the TRIM23/TBK1-mediated autophagy induction in WT and IFNAR1 KO cells.

→ **Reply:** Yes, our data indicate that TRIM23 acts antivirally in IFNAR1 KO cells due to autophagy activation (**Supplementary Fig. 2c**). Also, as described in our responses to the reviewer's earlier point (i.e., response #1) we have tested p62 degradation by ectopic TRIM23 or TBK1 expression in cells in which IFNAR signaling was blocked by anti-IFNAR2 antibody or not (of note, we did not use IFNAR1 KO MEFs because these cells are very hard to transfect). We found that ectopic TRIM23 or TBK1 effectively induced p62 degradation with or without IFNAR-signal inhibition to comparable levels (New **Supplementary Fig. 2f, g**).

5. It will be interesting to examine the viral replication in patient fibroblasts with the constitutively active TRIM23 mutant.

→ **Reply:** We thank the reviewer for this comment. In patient fibroblasts, which are TBK1-defective, virus restriction is compromised even in the presence of the constitutively active TRIM23 D/E mutant because p62 is not phosphorylated in TBK1-deficient cells and therefore cannot mediate efficient virus clearance.

Minor points:

1. Please show the TRIM23 protein level for knockout and knockdown confirmation.

→ **Reply:** Since the currently available commercial anti-TRIM23 antibodies do not reliably detect endogenous TRIM23 by Western blot analysis (of note, in our previous study we used a different antibody that has since been discontinued by the company), we confirmed the knockdown of TRIM23 by IF analysis, for which we have validated that the commercial TRIM23 antibody used in our current study works reliably (New **Supplementary Fig. 3a**).

2. Fig 3: the panels are incorrectly labeled and do not match the legends.

→ **Reply:** We have now corrected the figure legends to match the labeling with the figures.

Reviewer #3 (cGAS/STING) (Remarks to the Author):

Acharya and colleagues investigated the role of TRIM23 in cGAS-dependent antiviral autophagy. They report that the cGAS-STING-mediated classical autophagic pathway, in the context of HSV infection and dsDNA stimulation, requires TBK1-triggered TRIM23 S39 phosphorylation and subsequent autoubiquitination. The study addresses an interesting topic; however, the overall innovation of the research is limited, and the data presented are less compelling. The analysis appears somewhat cursory, and the interpretation of the data lacks thorough consideration. Thus, there are some important and interesting questions raised from this work that the manuscript does not address and would clearly increase the significance of the study.

→ **Reply:** We greatly thank the reviewer for the valuable comments, which we have addressed in the revised manuscript to improve our manuscript.

Major comments:

1. A hallmark of autophagic flux is the degradation of the selective autophagy receptor p62 and the conversion of LC3B-I to LC3B-II. The study appears somewhat inconsistent in its approach, lacking a unified standard. For instance, the evaluation of autophagic flux varies between p62 degradation, p62 phosphorylation, LC3I-II conversion via immunoblotting, and GFP-LC3B puncta formation. For example, in Figures 1E, 1J, and 1L, additional markers, such as LC3I-II conversion, are needed to better demonstrate that TRIM23 is critical for autophagic induction, beyond just p62 protein levels as shown in Figure 1H. Furthermore, there is inconsistency in the plasmid constructs used, alternating between myc-TRIM23 and flag-TRIM23.

→ **Reply:** We thank the reviewer for this important suggestion to strengthen our data. As suggested, we have repeated our experiments to assess endogenous LC3B-I-to-II conversion in various cell types in addition to measuring p62 abundance (New **Fig. 1g, 1i, 1k; Supplementary Fig. 5c, 5d, 5l, 5m**). Furthermore, we have analyzed LC3B puncta formation and p62 phosphorylation at S403 as additional read-outs (**Fig. 5F, g, I, j**), demonstrating the crucial role of TRIM23 in cGAS-STING-induced macroautophagy activation. Regarding the reviewer's comment about the TRIM23 plasmid, our TRIM23 construct actually has two tags, FLAG and myc, which we have now clarified in the Methods section (page 29, line 618-619).

2. Figure 4 relies heavily on overexpression systems, particularly in 293T cells. Given that these cells endogenously express TRIM23, evaluating the effects of endogenous TRIM23 expression is more convincing.

→ **Reply:** We thank the reviewer for raising this point. As suggested by the reviewer, we have now performed several additional experiments at the endogenous TRIM23 protein level in human dermal fibroblast (HDFs) that are physiologically relevant to HSV-1 infection. Specifically, we now show (1) S39 phosphorylation of endogenous TRIM23 following HSV-1 infection (New **Fig. 2f, g, h, i**) or dsDNA-ligand stimulation (New **Fig. 5b, c**); (2) endogenous TBK1 activation being crucial for mutHSV-1-induced endogenous TRIM23 S39 phosphorylation (New **Fig. 4f, g**); (3) co-localization of activated (i.e., S39-phosphorylated) TRIM23, TBK1 and STING at the endogenous protein level (New **Fig. 4h; Fig. 5k, l; Fig. 6a, b**); and (4) co-localization of activated endogenous TRIM23 with the Golgi early after STING activation, and with autophagosomes at later times after STING activation (New **Supplementary Fig. 6c-d**). Please also see our response to Reviewer #1.

3. Previously, Sparrer, K.M.J. et al. from the same research group demonstrated that TRIM23 mediates virus-induced autophagy through the activation of TBK1 (Nature Microbiology 2, 1543-1557 (2017)). In this study, they showed that TBK1 is the kinase responsible for phosphorylating and activating TRIM23, which is unsurprising. TRIM23 plays a pivotal role in the autophagic process triggered by various viruses, including HSV, EMCV, IAV, and SINV. In the context of HSV infection, it is logical that autophagy is triggered via cGAS. I am quite interested in exploring the function of the TBK1-TRIM23 axis in autophagy during IAV or SINV infection. Whether this process involves MAVS or TRIF is indeed an intriguing question. If the TBK1-TRIM23 axis is found to be a key regulator of autophagy upon activation by various upstream signaling pathways, it would significantly enhance the importance of this study.

→ **Reply:** We thank the reviewer for raising these points. As noted by the reviewer, our previous study (Sparrer et al., 2017) showed a pivotal role of TRIM23 in regulating TBK1-mediated p62 activation. In the current study, we found that TRIM23 is activated by TBK1-mediated

phosphorylation at S39 in its RING domain, suggesting an intricate feed-forward circuit of TBK1 and TRIM23 in autophagy. We have now performed a series of additional experiments to dissect the molecular details of this feed-forward cycle, which led to [one additional main figure and one additional supplementary figure](#) (New **Fig. 6 and Supplementary Fig. 6**). Our new data show that TRIM23 C34A and the GTPase-dead mutant K458I were effectively phosphorylated by TBK1 (New **Supplementary Fig. 6b**), while TRIM23 Δ ARF (TBK1-binding-deficient) and TRIM23 Δ RING (lacking S39) were not, suggesting that TBK1 acts as upstream kinase for TRIM23 leading to its activation. Upon HSV-1 infection or dsDNA-induced cGAS-STING activation, STING, TBK1 and TRIM23 form a trimolecular complex which promotes the phosphorylation of TRIM23 by TBK1 at the Golgi apparatus (New **Fig. 6a, b; Supplementary Fig. 6c, d**); this phosphorylation event then triggers TRIM23 auto-ubiquitination of the ARF domain promoting GTP-GDP cycling; GTPase activity facilitates higher-order assembly of bound TBK1 as well as trafficking of the activated TRIM23-TBK1 complex to autophagosomes, ultimately leading to p62 phosphorylation by TBK1 (Sparrer et al., 2017; PMID: 28871090). We have now also discussed in more detail this proposed feed-forward circuit in the Discussion section (page 26, lines 550-556). Please also see our responses to Reviewer #1 (major point #4) and Reviewer #2 (major point #3), who have raised the same point.

Furthermore, our previous study (Sparrer et. al., 2017) indicated that TRIM23 regulates autophagic flux to multiple viruses including HSV-1 and the RNA viruses IAV, EMCV and SINV. Despite TBK1 being known to be activated by both RNA viruses and DNA viruses (of note, this has only been shown for TBK1's role in the IFN response), we found that only HSV-1, but not the RNA viruses IAV, SINV or VSV, induced phosphorylation of endogenous TRIM23 at S39 (**see Figure below**). Consistent with this observation, DNA ligands (as well as cGAMP), but not RNA ligands, induced the phosphorylation of TRIM23 at S39 (New **Fig. 5b, c**). We believe this is an interesting finding which suggests that unique molecule(s) activated by DNA viruses (via cGAS STING), but not by RNA viruses, induce TBK1's activity for eliciting TRIM23-mediated autophagy. How RNA viruses activate TRIM23 for eliciting autophagy will be an interesting future research direction; we have discussed this in the Discussion section (Page 23, Line 483-495).

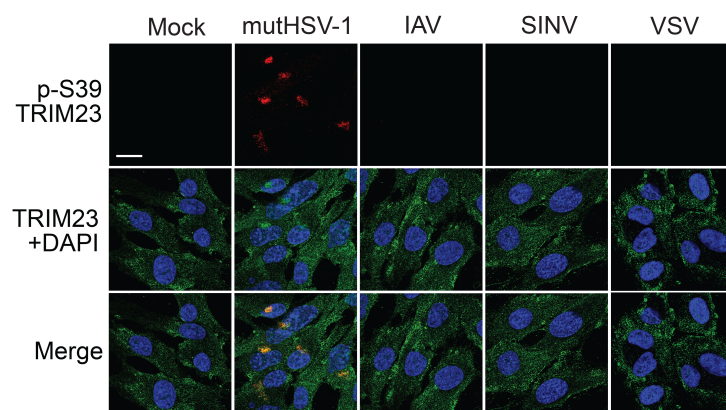


Figure included for reviewers: S39 phosphorylation of endogenous TRIM23 in HDF cells that were either mock-treated or infected with mutHSV-1 (MOI 5), IAV (MOI 10), SINV (MOI 10) or VSV (MOI 2), determined at 16 h post infection by immunostaining with the indicated antibodies and confocal microscopy analysis. DAPI, nuclei (blue). Scale bar, 20 μ m.

4. It is intriguing that TRIM23 specifically mediates autophagy in the cGAS-STING pathway, but not in the RLR or TLR pathways. Given that key adaptors in the DNA sensing, RLR, and TLR pathways—namely STING, MAVS, and TRIF—share a conserved motif that interacts with TBK1 and requires TBK1 phosphorylation to activate downstream signaling, could you provide a detailed explanation for why the TBK1-TRIM23 axis is a critical regulator of autophagy specifically in the cGAS pathway? What are the potential mechanisms underlying this specificity?

→ **Reply:** We thank the reviewer for these insightful comments. As noted by the reviewer, stimulation of cells with DNA ligands (or cGAS-STING overexpression), but not with RLR ligands (or RLR overexpression), potently elicited S39 phosphorylation of TRIM23 (**Fig. 5b, c**), hinting at a unique signaling event and/or mediators downstream of cGAS (but not RLR activation) that facilitate TRIM23 activation by TBK1. In line with our own observations, it was also noted by others that DNA ligands are more potent stimulators of autophagy as compared to RNA ligands (e.g., Gui et al., *Nature* 2019, PMID: 30842662). Why TBK1 activation downstream of cGAS-STING signaling (but not RLR or TLR signaling) mediates TRIM23 S39 phosphorylation is a very interesting question, which warrants further investigation and, we believe, is outside the scope of the current study. To still address the reviewer's point, we have now further discussed this in the Discussion section of our revised manuscript (Page 23, Line 483-495).

5. Additionally, could you elaborate on the nature of the binding relationship between the STING-TBK1-TRIM23 complex? Specifically, how does TBK1 interact with STING and TRIM23 within this complex, and what role does this interaction play in mediating cGAS-STING-induced autophagy? Understanding the structural and functional dynamics of this interaction could provide deeper insights into the specificity of the TBK1-TRIM23 axis in STING-triggered autophagy regulation.

→ **Reply:** As suggested by the reviewer, we examined the binding relationship between the STING-TBK1-TRIM23 complex. Through IF analysis assaying endogenous proteins, we found that p-S39-TRIM23, p-S172-TBK1 and STING form a complex after HSV-1 infection or DNA ligand stimulation (New **Fig. 6a, b**). Biochemical mapping studies showed that TRIM23 binds to the cyclic dinucleotide-binding domain (CBD) of STING (New **Fig. 6c, d**). Of note, we have previously shown that TRIM23 interacts using its ARF domain with the N-terminal kinase domain (KD) of TBK1 (Sparrer et al., 2017, PMID: 28871090). Complementing these IF and co-IP data, we also performed PLA to assess the subcellular localization of S39-phosphorylated TRIM23. We observed a close spatial proximity of endogenous p-S39-TRIM23 with the Golgi marker GM130 at early times after cGAMP stimulation (New **Supplementary Fig. 6c, d**). At later times, however, p-S39-TRIM23 showed spatial proximity with ATG16, indicating TRIM23's localization to autophagosomes (New **Supplementary Fig. 6c, d**). These observations suggest that within few hours after cGAS-STING activation, TRIM23 is phosphorylated by TBK1 at the Golgi apparatus; later then, the activated TRIM23-TBK1 complex translocates to autophagosomes, ultimately facilitating TBK1-mediated phosphorylation of p62.

6. The patient samples with HSE provide strong evidence for the correlation between TBK1 and TRIM23. I am curious whether functional mutations in TRIM23 are present in HSE patients. Identifying such mutations would provide more direct evidence of TRIM23's importance in the disease context.

→ **Reply:** It would be very interesting to explore if there are any functional mutation(s) in TRIM23 in HSE patients or other autophagy deficiencies. However, no such mutation(s) have been reported as of now.

Minor comments:

1. The current title seems out the scope of the study. To ensure accuracy, I suggest revising the title to “TRIM23 Mediates cGAS-Induced Autophagy in Anti-HSV Defense”.

→ **Reply:** We have changed the title as suggested.

2. More referenced is needed in line 50-52:cGAS-STING activation induces “classical” p62/SQSTM1-mediated autophagy as well as “non-canonical” autophagy or CASM (conjugation of ATG8 to single membranes)10, 11, 12, 13, 14. (PMID: 36872914,PMID: 19926846,PMID: 34453126,PMID: 37535724).

→ **Reply:** As requested, we have now cited these additional references (page 3, line 52-54).

3. In reviewing Figures 1C, 1G, and 1K, it is not clear on what basis the specific viral proteins were chosen to indicate viral replication. The selection criteria for these viral proteins should be clearly stated, as the current presentation appears somewhat arbitrary. Additionally, Figure 1H shows a test for viral titer instead of RT-qPCR.

→ **Reply:** We thank the reviewer for this comment. We have now analyzed additional genes from these RT-qPCRs and now consistently show at least two viral genes (i.e., ICP0 and ICP8) in all our RT-qPCR data sets (New **Fig. 1c, 1f, 1j**). We also have now performed RT-qPCR analysis of ICP0 and ICP8 for the data (**Fig. 1h**) where we previously only showed plaque assay (New **Supplementary Fig. 1g**). For some experiments, we show viral titers by plaque assay in addition to RT-qPCR (**Fig. 1b, 1h**), which will give absolute values of virus particles.

4. In Supplementary Figures S2A-C, the selection of representative ISGs appears limited. Including a broader range of ISGs or providing transcriptomic data could offer a more comprehensive view of the ISG response. This additional information would strengthen the interpretation of the data and provide a more thorough understanding of the gene expression changes associated with the experimental conditions.

→ **Reply:** As suggested, we added additional ISGs (New **Supplementary Fig. 2a**), which have strengthened our observation.

5. The figure legends (3d-f) need to be reordered for clarity.

→ **Reply:** We apologize for this mistake. We corrected the figure legends to match the figure labeling.

6. In Figure 3H, in addition to presenting the viral titer, it would be beneficial to include data on HSV-specific antibodies to demonstrate the viral potency.

→ **Reply:** In this figure, we showed a representative dataset of TRIM23 WT and mutant protein expression right before cells were infected with HSV-1, which we have clarified in the figure legend (**Fig. 3h**).

7. In Figure 4A, a negative control is missing to demonstrate the specificity of the phosphorylated bands.

→ **Reply:** As suggested, we have repeated this experiment (New **Fig. 4a**) by including a negative control (i.e., no TRIM23 expression) in the first lane, and also included quantification data in the revised manuscript (New **Supplementary Fig. 4b**).

8. In Figures 3C, 4J, and 5D, the statistical methods used are inconsistent, which makes the analysis appear arbitrary.

→ **Reply:** We now consistently use Student's *t*-test for all data sets. Specifically, we have now used Student *t*-test (two-tailed) for data with a sample size of $n \leq 6$. For larger data sets (e.g., PLA assays, GFP-LC3 puncta counting, and colocalization quantification by Pearson's correlation coefficient), we have used a more stringent version of Student *t*-test with Welch correction.

9. In all the virus infection experiments, including those in Western blot analyses, there is a lack of data on HSV-related protein expression to confirm the consistency of the viral infection, as seen in Figure 5A.

→ **Reply:** As suggested, we have now included the HSV-1 protein expression blot (Revised **Fig. 5a**). Of note, PRRs signaling, beside regulation of autophagy, modulates viral replication via regulation of cytokine responses. To assess the role of PRRs specifically in autophagy, it is crucial to ensure that the different samples/conditions are equally infected. Therefore, in Figure 5A, we used a relatively high MOI (MOI 10) and measured HSV-1 proteins by western blot to ensure that all the samples were equally infected (page 14, line 293-295).

10. In Figure 5A, the expression levels of LC3I/II need to be provided.

→ **Reply:** As suggested, we have now assessed LC3B-I-to-II conversion for this experiment (New **Supplementary Fig. 5c**). In addition, we now also demonstrate that mutHSV-1 induces autophagy via STING activation by analyzing p62 degradation and LC3B-I-to-II conversion as readouts (New **Supplementary Fig. 5d**).

11. In Figure 4B, several key details are missing: there is no reference to internal controls in the whole cell lysate (WCL), nor are the expressions of Flag and phosphorylated proteins provided. Additionally, the expression of HA and Myc in the immunoprecipitation (IP) experiments is not shown. It is also unclear whether TBK1 interacts with TRIM23. Similar issues are noted in Figures 4C, 4F-H, 5B and 6B.

→ **Reply:** As suggested, we have included a reference control (actin) in the WCLs in all these experiments (Improved **Fig. 4b, c, e, i, j**). Of note, we removed the myc-PINK1 related data from **Fig. 4b** as suggested by Reviewer #1. Our p-S39-TRIM23 antibody does not work well in WCL (it works well only after IP; or in IF analysis as shown in various figures of our revised manuscript). We have added data on the TRIM23-TBK1 interaction blot in our revised manuscript (**Supplementary Fig. 6a**). Furthermore, in our previous study (Sparrer et al., 2017, PMID: 28871090) we mapped the interaction between TRIM23 and TBK1, which showed that TRIM23 via its ARF domain interacts with the N-terminal kinase domain (KD) of TBK1.

12. Figure 4D, 4F lacks a negative control.

→ **Reply:** As suggested, we have repeated these two experiments by including a negative control in the first lane (New **Fig. 4d, e**).

13. Usually, p62 phosphorylation correlates with changes in p62 levels. However, this correlation does not appear to hold in Figures 5G and 5H. Please clarify.

→ **Reply:** p62 is degraded upon autophagy activation, which makes it difficult to reliably assess p62 phosphorylation induced by some autophagy inducers. Therefore, in these experiments we inhibited p62 degradation by blocking autophagic flux using a combination of Pepstatin A and E64D (10 µg/mL each). We have now added these experimental details to the respective figure legends (**Fig 5i, j**) and Methods (page 33, line 714-719).

14. Please provide LC3 expression in Figure 5G.

→ **Reply:** As noted above, this experiment (**Fig. 5i** in the revised manuscript) was designed to assess p62 phosphorylation. We have assessed in parallel LC3B-I-to-II conversion which is shown in **Fig. 5h** of the revised manuscript.

15. Typically, the level of autophagy is assessed by comparing the ratio of LC3I to LC3II. However, in Figure 5H, the transition between LC3I and LC3II does not support the conclusion presented.

→ **Reply:** We repeated this experiment and now show both LC3B-I-to-II conversion and p62 degradation (New **Supplementary Fig. 5L**).

16. CCL5 data is missing in S1C.

→ **Reply:** We have corrected this in the revised figure legends.

17. S4B LC3I/LC3II ratio is quite different from 5H.

→ **Reply:** Different cell types have different basal autophagy levels, which is reflected in a different LC3-I-to-II ratio. For example, while primary fibroblasts have low basal autophagy (reflected in high levels of LC3-I, and minimal levels of LC3-II), HEK293T cells have a quite high basal autophagy (already strong LC3-II levels without any specific autophagy stimulation; and weak LC3-I band). This phenomenon has been noted in many publications (e.g., Gui et al., 2019, Nature, PMID 30842662). We believe that our data in HEK293T still clearly show that cGAS-STING robustly induces LC3B-I-to-II conversion, as compared to the other proteins included as controls.

18. Where is the LC3I band in S4G?

→ **Reply:** We have repeated this experiment (Improved **Supplementary Fig. 5j**), which now shows both bands for LC3B. Please see also our comment above on different LC3-I/II ratios in different cell types.

19. TBK1-p should be provided in figure 6A.

→ **Reply:** As suggested, we have added p-S172-TBK1 and total TBK1 blots for these data (Improved **Fig. 7a**).

20. Rephrase lines 127-129, as A549 cells have not shown data on p62 degradation. The text description does not match the data, and the conclusion appears hasty and lacking in rigor.

→ **Reply:** We have now modified the text.

21. Please provide references for the GFP-LC3B puncta assay.

→ **Reply:** We have now added references (Klionsky et al., 2021, PMID 33634751; Sparrer et al., 2017, PMID 28871090) for the GFP-LC3B puncta assay.

Reviewer #4 (ECR) (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

→ **Reply:** We thank the reviewer for co-reviewing our manuscript.

Reviewer #5 (ECR) (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

→ **Reply:** We thank the reviewer for co-reviewing our manuscript.

Point-by-point response to reviewer's comments (Revised manuscript)

We would like to thank all reviewers for evaluating our revised manuscript and the very positive comments. We have addressed the additional minor points raised by the reviewers, including the suggestion (by reviewer #1 and #3) to include a figure displaying a working model of the cGAS-STING-TBK1-TRIM23 axis in autophagy.

Reviewer #1 (Remarks to the Author):

In the revised manuscript, Acharya et al. addressed most of the reviewers' comments and questions and they improved their figures significantly. The co-reviewer and I are both in support of this manuscript's acceptance to Nature Communications. The following minor comments should be addressed to improve the final manuscript.

→ **Reply:** We thank the reviewer and co-reviewer for evaluating our revised manuscript and for providing these additional comments, which we have fully addressed.

Minor Comments:

There is no statistical information presented in the graphs of Supplementary Figures S1f–i, S2b–e, S4a–b, S5a and S5k, despite the figure legend stating that statistical tests were conducted. Please clarify this and indicate whether only significant values are shown or provide the relevant statistical details.

→ **Reply:** We thank the reviewer for indicating this. We have now added statistical details in the graphs of Supplementary Figures S1f–i, S2b–e, S4a–b, S5a and S5k and described these in the respective figure legends.

Line 164: I think the authors mean “dispensable” instead of “indispensable”.

→ **Reply:** We thank the reviewer for indicating this mistake, which we have now corrected.

While not required, a diagram displaying the authors' model for the STING-TBK1-TRIM23 feed-forward loop would be helpful.

→ **Reply:** As suggested, we have now included a diagram displaying the working model for the cGAS-STING-TBK1-TRIM23 axis in autophagy (New Supplementary Fig. 7d).

Reviewer #2 (Remarks to the Author):

The authors have addressed the previous concerns satisfactorily using new expts.

→ **Reply:** We thank the reviewer for positively evaluating our revised manuscript.

Reviewer #3 (Remarks to the Author):

I appreciate the authors' efforts in addressing my previous comments. The revisions have significantly improved the manuscript, and I am satisfied with the responses provided. To enhance the clarity and impact of your manuscript, I strongly recommend including a model figure that summarizes the key findings of your study and their implications for the cGAS-STING-autophagy axis. This figure should visually depict the proposed molecular mechanisms, including the phosphorylation of TRIM23 by TBK1, the formation of the STING-TBK1-TRIM23 complex, and the subsequent activation of autophagy. The model should highlight the novel aspects of your work, such as the unique role of TRIM23 S39 phosphorylation and the feed-forward loop between TBK1 and TRIM23. Additionally, the figure could include a discussion of the broader implications of your findings, such as potential therapeutic targets or connections to autophagy-related diseases. Please ensure the figure is visually clear and accompanied by a concise legend to aid reader comprehension.

→ **Reply:** We thank the reviewer for the positive comments and additional suggestions. As suggested, we have now included a diagram displaying the working model for the cGAS-STING-TBK1-TRIM23 axis in autophagy in the revised manuscript (New Supplementary Fig. 7d).

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

→ **Reply:** We thank the reviewer for co-reviewing our revised manuscript.