

Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity

Corresponding Author: Professor Xiao Yu

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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)

Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity (Nature Communications manuscript NCOMMS-25-12518-T)

The authors demonstrated that APT2 regulates TBK1 through depalmitoylation, modulating IFN-I signaling during Plasmodium infection. This study provides remarkable insights into TBK1 regulation; however, several points require revision as outlined below.

Major

1. Clarifying the Effect of Palmitoylation on TBK1 Phosphorylation

The authors demonstrated that TBK1 phosphorylation induced by Plasmodium infection is reduced following palmitoylation inhibition (Fig. 1h-n, Fig. 4b). Plasmodium infection leads to a decrease in TBK1 palmitoylation (Fig. 2a). Moreover, it was substantiated that Plasmodium infection upregulates APT2 expression (Fig. 7a, b). Notably, TBK1 phosphorylation decreases following APT2 overexpression (Fig. 4q). Taken together, the authors suggest that this mechanism results from APT2-mediated negative feedback regulation.

However, this raises the question of whether the reduction in phosphorylated TBK1 is due to the decrease in total TBK1 levels caused by negative feedback, rather than a direct effect of reduced palmitoylation. If TBK1 phosphorylation decreases as part of a feedback mechanism to prevent excessive IFN-I activation, then it may be independent of palmitoylation. Instead, the reduction in TBK1 phosphorylation could be an indirect effect of TBK1 degradation rather than a direct effect of palmitoylation loss.

Therefore, it is necessary to distinguish whether the observed decrease in TBK1 phosphorylation is due to palmitoylation loss or a result of total TBK1 degradation.

- Assess whether TBK1 phosphorylation decreases even when total TBK1 protein levels are maintained. To address this, TBK1 phosphorylation levels should be tested after treatment with proteasome/autophagy inhibitors (MG132, Baf A1) in combination with palmitoylation inhibitors (2-BP, APT2 overexpression).
- Examine whether TBK1 phosphorylation is similarly reduced in the C292S mutant (which cannot be palmitoylated). Examining TBK1 phosphorylation levels in the C292S mutant will help determine whether palmitoylation directly regulates phosphorylation.

2. Contrasting Roles of K48- and K63-Linked Ubiquitination in TBK1 Degradation

The authors demonstrated that APT2 depalmitoylates TBK1, increases K48-linked ubiquitination, and promotes autophagy-mediated degradation. However, TBK1 shows decreased K63-linked ubiquitination (Fig. 6a).

Previous studies indicate that K48-linked ubiquitin chains target proteins for proteasomal degradation and are typically considered canonical ubiquitination signals. In contrast, K63-linked ubiquitination does not target proteins for proteasomal degradation but is instead involved in autophagy.

Thus, it is unclear why APT2 increases K48-linked ubiquitination while accompanied by a decrease in K63-linked ubiquitination. Further investigation is required to explain how TBK1 undergoes autophagic degradation despite the reduction in K63-linked ubiquitination.

- Compare TBK1 degradation in MG132-treated cells with Lactacystin or Epoxomicin (proteasome-specific inhibitors) to determine if degradation is proteasome-independent.
- Generate TBK1 K48R or K63R mutants to analyze how each ubiquitination type affects TBK1 degradation and autophagy.

3. Investigating the Role of TBK1 Palmitoylation in Ubiquitination During Plasmodium Infection

Fig. 1b and Fig. 7f show that the TBK1-IFN-I signaling pathway initially suppresses infection, but as Plasmodium immune evasion mechanisms activate, negative feedback leads to TBK1 degradation, ultimately increasing parasitemia again. The authors propose that APT2 depalmitoylates TBK1, increases K48-linked ubiquitination, and promotes TBK1 degradation via autophagy. This raises the question of whether TBK1 palmitoylation suppresses K48-linked or K63-linked ubiquitination during the early stages of infection, before negative feedback is initiated.

Thus, it is important to determine how TBK1 ubiquitination patterns (K48-linked, K63-linked) change when ZDHHC9 is overexpressed, thereby increasing TBK1 palmitoylation.

- Investigate whether ZDHHC9 overexpression alters TBK1 ubiquitination patterns (K48/K63).
- Examine the temporal changes in TBK1 ubiquitination patterns during Plasmodium infection.

4. Evaluating the Impact of APT2 on IRF3 Phosphorylation and Nuclear Translocation During Plasmodium Infection

The authors confirmed that Plasmodium infection leads to increased APT2 expression (Fig. 7a, b) and TBK1 instability, as demonstrated by the increased TBK1 degradation in CHX chase assays (Fig. 5e, f). Considering that APT2 expression increases during Plasmodium infection, and that TBK1 degradation should lead to reduced IRF3 phosphorylation or nuclear translocation, it is necessary to experimentally confirm whether APT2-mediated negative feedback reduces IRF3 phosphorylation and nuclear localization.

- Assess changes in IRF3 phosphorylation levels following APT2 overexpression.
- Examine IRF3 nuclear translocation following APT2 overexpression using confocal microscopy or nuclear fractionation.

Minor

1. The legend for the 'Uninfected group' is missing in Fig. 1g.

Reviewer #2

(Remarks to the Author)

The manuscript by Han et al. clearly demonstrates that TBK1 palmitoylation is involved in type-1 interferon production in models of malaria infection. This is antagonized by APT2, which depalmitoylates TBK1 leading to its degradation via autophagy. This is a very strong manuscript that could interest experts in immune responses against parasitic infections. However, a few additional experiments would broaden the scope of this manuscript, making it more relevant to the field of innate immunity.

Major points:

- TBK1 is also an important kinase in TRIF-mediated signaling (Fitzgerald et al., Nature Immunology, 2003), a signaling pathway that is activated upon exposure to Plasmodium (Couper et al., Plos Pathogens, 2010) (Mantel et al., Cell Host and Microbe, 2013) (Zhang et al., Exp Parasitology, 2016). So, it is possible that the observed effects are TLR-mediated, not only STING/MAVS as suggested. Further experimentation to establish which pathway(s) is involved in TBK1 palmitoylation/depalmitoylation would give us a clearer understanding of the signaling events involved.
- The manuscript focused on Plasmodium parasites and established clear links between TBK1 palmitoylation/depalmitoylation with ZDHHC9/APT2 in this context. Are these players the same in other contexts (either other infections or in cells stimulated with "clean" agonists)?
- Figure 2k is not very clear. ZDHHC9 has a FLAG-tag, but what about TBK1? Additionally, HEK expression systems can lead to high expression levels, forcing interactions that don't necessarily occur at physiological levels. A control using an unrelated protein would strengthen this figure.
- Is there any direct evidence that APT2 and TRIM21 physically interact? (Co-IP, colocalization, etc?)

Minor points:

- (Introduction): There isn't a clear consensus on whether hemozoin itself activates pattern recognition receptors, as it is likely that hemozoin needs to be "contaminated" with other molecules to stimulate PRRs (Parroche et al, PNAS, 2007).
- (Introduction): In addition to STING/MAVS, TBK1 is also activated via TRIF (Fitzgerald et al., Nature Immunology, 2003).
- (Introduction): NF-kB and IRF3/7 are transcription factors activated by various signaling pathways such as TLRs, IL-1Rs, cGAS/STING, etc. I believe that saying that NF-kB and IRF3/7 are signaling pathways is conceptually inaccurate.
- (Results): (Figure 1) TBK1 phosphorylation goes down, but so does the total protein levels. Are there differences in the p-TBK1/TBK1 ratio.
- (Results): "Furthermore, Co-IP experiments showed that APT2 physiologically interacts with TBK1 (Extended Data Fig. 3e)". This refers to an experiment using HEK cells transfected with various proteins, it is not accurate to call this a physiological condition.
- (Results): Differences in Figure 5g are not visually obvious. Densitometric quantification might make this point a bit more convincing.
- (Methods): Part of "Western blotting and Co-immunoprecipitation" needs to be rewritten.

Reviewer #3

(Remarks to the Author)

The manuscript entitled "Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity" presents an in-depth study on the palmitoylation of the TBK1 protein and its impact on malaria immunity. While the study is interesting and potentially impactful, there are several serious concerns that need to be clarified before the manuscript can be considered for publication.

Major concerns

- What is the rationale for focusing on type-I interferons in the context of malaria infection? The manuscript does not elaborate on why this specific pathway was chosen over others, despite the fact that numerous immune signaling pathways are dysregulated during malaria infection. Since type-I interferons are more commonly associated with antiviral responses, the study should explain their relevance in malaria pathogenesis. Including supporting data or references that highlight the role of type-I interferons in Plasmodium infections would strengthen the justification for this focus.
- The authors should clarify why only TBK1 and IRF3 were examined in the Western blot analyses. Given that both STING and MAVS function upstream of TBK1, it would be important to assess their involvement to better understand the signaling cascade. Additionally, since TBK1 also regulates the NF- κ B pathway, examining key NF- κ B markers such as phosphorylated I κ B α or p65 would provide a more comprehensive view of TBK1's role in the immune response to malaria infection. Expanding the analysis to include these upstream and downstream components would strengthen the study's mechanistic insights.
- The authors should provide a more detailed explanation of the infection model used in their experiments. In the study, PBS was used as a control, while iRBCs were used for infection. However, it is important to clarify how this model accurately mimics natural infection conditions and ensures that the observed immune response is due to the infection itself rather than simply the presence of foreign nucleic acids. Was the parasite live in iRBCs? Additional controls, such as uninfected RBCs or heat-killed iRBCs, could help distinguish between infection-specific immune activation and a general response to foreign nucleic acids.
- Another important consideration is whether the experiments with N67 gDNA and RNA included appropriate control DNA or RNA. This is crucial because nucleic acids, in general, can stimulate immune responses. Without proper controls it would be difficult to determine whether the observed immune activation was specific to malaria or simply a general response to foreign nucleic acids. Were such controls included in the experiments?
- The major concern about the discovery is the lack of transparency of the RNA-seq experiments. How many replicates are used? The heatmap plot shows that a single replicate is only used for each group, and it is strongly recommended to have triplicates to infer the results with more confidence. Maybe a PCA plot can better represent the distribution and number of samples. It is strongly recommended that the raw data be deposited in public nucleotide archives like SRA.
- Another major concern is the approach behind the identification of ZDHHC9. It is mentioned in the figure 2J that at the RNA level, all the family members are dysregulated. Some genes are dysregulated to almost zero. Is it normalized in comparison with uninfected control? If the genes are this much dysregulated upon infection, why are they not present in the RNA-seq results? Maybe a heatmap of these family members in RNA-seq can explain this better. Protein level dysregulation can be showed for better clarity.
- The mere reduction of the ZDHHC proteins may not correlate with the palmitoylation level of TBK1. The depalmitoylation by increasing the level of APT2 can also be the reason for the reduction of palmitoylation during malarial infection. A coimmunoprecipitation study with the whole family of ZDHHC family members with TBK1 could have shed light on this scenario.
- It is mentioned that the treatment with APT2 inhibitor ML349 enhances the anti-malarial immune response by elevating the palmitoylation level of TBK1 and increasing IFN- γ . However, the inhibition of APT2 by ML349 can affect all of its interactors, not only TBK1. For instance, the upstream adapter MAVS is also affected by ML349, as per various studies.

Minor Concerns

- In several figures (fig. 1P, extended fig 1i, 1j), the infection with N67 iRBCs is inducing more response than the N67 RNA or N67 gDNA alone. How is it possible and the authors should shed light on this matter and explain the infection model better. ISRE luc is lower than IFN luc in few experiments (Extended Data Fig. 2d). What could be the potential reason?
- The authors should provide data on the knockdown efficiency of siRNAs and shRNAs, as this is crucial for validating the reliability of their gene silencing experiments. Currently, the manuscript does not include any evidence—neither in the main figures nor in the supplementary figures—demonstrating the extent of knockdown at the mRNA or protein level.
- Please clarify whether the enzymatically inactive mutants ZDHHC9 (C169S) and APT2 (S122A, C2S) have been previously characterized in published studies. If these mutants have been established in earlier research, proper citations should be provided to support their validity.
- The authors should ensure that the cell lines used in each experiment are clearly indicated in the figures, as was done in the initial figures of the manuscript. Consistently labeling the cell lines throughout the figures would improve clarity and help readers accurately interpret the results.
- The authors should correct the inconsistency between the figure legend, the main manuscript description, and the actual figure. In the legend for Fig. 4e and 4f, as well as in the manuscript text, both IFN β -luc and ISRE-luc are mentioned. However, in the figure itself, only IFN β -luc is shown. The authors should clarify whether ISRE-luc data were included and, if not, revise the text accordingly to accurately reflect the data presented in the figure.
- The authors can include an explanation of how parasitemia was calculated, as this is not mentioned in the Methods section. Providing details on the methodology—such as whether parasitemia was assessed by Giemsa staining, flow cytometry, or qPCR—is essential for reproducibility and proper interpretation of the results.

(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)

My comment has been resolved.

Reviewer #2

(Remarks to the Author)

The authors successfully addressed my concerns. I believe this is a strong manuscript.

Reviewer #3

(Remarks to the Author)

In the revised manuscript titled "Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity," the authors have adequately addressed all the concerns raised during review. The manuscript is in a strong and polished form, with the content, structure, and presentation meeting the standards required for publication.

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.

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Responses to Reviewer #1:

Reviewer #1:

*Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity (Nature Communications manuscript NCOMMS-25-12518-T)
The authors demonstrated that APT2 regulates TBK1 through depalmitoylation, modulating IFN-I signaling during Plasmodium infection. This study provides remarkable insights into TBK1 regulation; however, several points require revision as outlined below.*

Response: We thank the reviewer very much for the insightful comments of our manuscript and are happy to address all concerns raised.

Major

1. Clarifying the Effect of Palmitoylation on TBK1 Phosphorylation

The authors demonstrated that TBK1 phosphorylation induced by Plasmodium infection is reduced following palmitoylation inhibition (Fig. 1h-n, Fig. 4b). Plasmodium infection leads to a decrease in TBK1 palmitoylation (Fig. 2a). Moreover, it was substantiated that Plasmodium infection upregulates APT2 expression (Fig. 7a, b). Notably, TBK1 phosphorylation decreases following APT2 overexpression (Fig. 4q). Taken together, the authors suggest that this mechanism results from APT2-mediated negative feedback regulation.

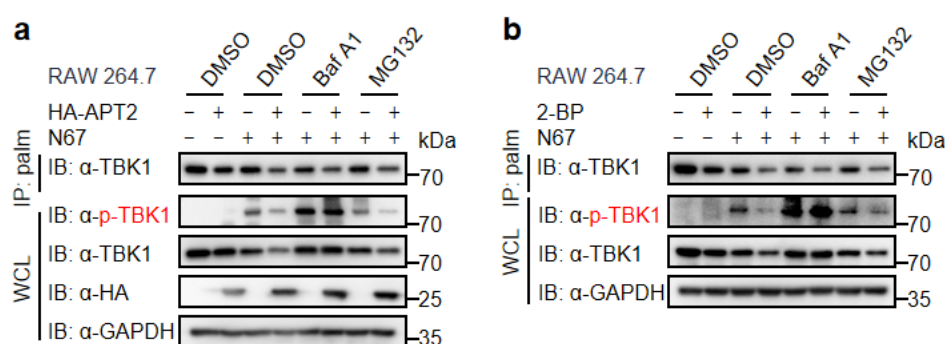
However, this raises the question of whether the reduction in phosphorylated TBK1 is due to the decrease in total TBK1 levels caused by negative feedback, rather than a direct effect of reduced palmitoylation. If TBK1 phosphorylation decreases as part of a feedback mechanism to prevent excessive IFN-I activation, then it may be independent of palmitoylation. Instead, the reduction in TBK1 phosphorylation could be an indirect effect of TBK1 degradation rather than a direct effect of palmitoylation loss.

Therefore, it is necessary to distinguish whether the observed decrease in TBK1 phosphorylation is due to palmitoylation loss or a result of total TBK1 degradation.

- *Assess whether TBK1 phosphorylation decreases even when total TBK1 protein levels are maintained. To address this, TBK1 phosphorylation levels should be tested after treatment with proteasome/autophagy inhibitors (MG132, Baf A1) in combination with palmitoylation inhibitors (2-BP, APT2 overexpression).*
- *Examine whether TBK1 phosphorylation is similarly reduced in the C292S mutant (which cannot be palmitoylated). Examining TBK1 phosphorylation levels in the C292S mutant will help determine whether palmitoylation directly regulates phosphorylation.*

Response: We sincerely appreciate this insightful question.

(1) In response to your suggestion, we treated cells with the autophagy inhibitor Baf A1 and the proteasome inhibitor MG132, both in combination with palmitoylation inhibitors. Our experimental results demonstrated that APT2 and 2-BP effectively decreased both the palmitoylation and phosphorylation levels of TBK1. Notably, the autophagy inhibitor Baf A1 effectively blocked APT2 or 2-BP induced TBK1 degradation and rescued the phosphorylation levels of TBK1 (**New Figure 1**, presented as **Fig. 5i** and **Supplementary Fig. 5d**). These findings indicate that the reduction in TBK1 phosphorylation is directly caused by autophagic degradation mediated by depalmitoylation of TBK1. Furthermore, while we confirmed that autophagy inhibitor Baf A1 successfully blocked APT2- or 2-BP-induced TBK1 degradation, it failed to restore TBK1 palmitoylation levels. This crucial observation suggests that in the context of APT2 or 2-BP treatment, the depalmitoylation event of TBK1 occurs upstream of the autophagic degradation pathway and serves as a necessary prerequisite for its targeting to autophagic degradation. Consequently, even when autophagic degradation is inhibited, the palmitoylation level of TBK1 remains low due to the prior depalmitoylation events, which is irreversible. Relevant data have been added to the manuscript (Lines 270-272, Page 9).



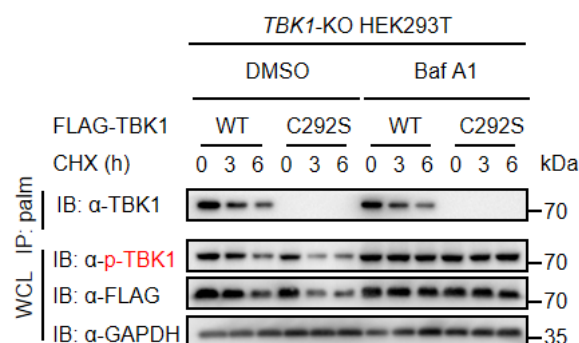
New Figure 1, presented as **Fig. 5i** and **Supplementary Fig. 5d** in the revised manuscript.

(a) RAW 264.7 cells were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with freeze-thawed iRBCs for 12 h and then DMSO (control), bafilomycin A1 (Baf A1) (0.2 μ M), or MG132 (10 μ M) treatment for 6 h. Cell lysates were collected for ABE assay and immunoblot analysis.

(b) RAW 264.7 cells were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with freeze-thawed iRBCs for 12 h and then DMSO (control), Baf A1 (0.2 μ M), or MG132 (10 μ M) treatment for 6 h. Cell lysates were collected for ABE assay and immunoblot analysis.

(2) Consistent with its accelerated degradation (as shown in **Fig. 5e** and **5f**), the palmitoylation-deficient TBK1-C292S mutant exhibited significant lower levels of activated p-TBK1 compared to wild-type TBK1 (**New Figure 2**, presented as **Supplementary Fig. 5e**). Importantly, treatment with autophagy inhibitors, which stabilize TBK1-C292S protein levels, completely abrogated the decrease in p-TBK1.

These findings demonstrate that the observed reduction in p-TBK1 in TBK1-C292S mutant is primarily due to increased protein degradation, rather than a direct effect of palmitoylation loss on phosphorylation. Palmitoylation of TBK1 plays a crucial role in sustaining its phosphorylation status by maintaining protein stability. Loss of palmitoylation leads to TBK1 degradation via autophagy, consequently depleting the pool of TBK1 available for phosphorylation (Lines 272-276, Page 9).



New Figure 2, presented as **Supplementary Fig. 5e** in the revised manuscript. *TBK1*-KO HEK293T cells were reconstituted with plasmids encoding wild-type FLAG-TBK1 or the FLAG-TBK1 C292S mutant for 24 h. Subsequently, the cells were treated with CHX (100 mg/mL) at specified time points, along with DMSO (as a control) and Baf A1 (0.2 μ M) treatment for 6 h. Cell lysates were then collected for ABE assay and immunoblot analysis.

Thus, TBK1 palmitoylation provides protection against autophagic degradation. *Plasmodium* infection-induced upregulation of APT2 removes this protective modification, resulting in TBK1 degradation, which ultimately leads to decreased phosphorylation of its target proteins.

2. Contrasting Roles of K48- and K63-Linked Ubiquitination in TBK1 Degradation

The authors demonstrated that APT2 depalmitoylates TBK1, increases K48-linked ubiquitination, and promotes autophagy-mediated degradation. However, TBK1 shows decreased K63-linked ubiquitination (Fig. 6a).

Previous studies indicate that K48-linked ubiquitin chains target proteins for proteasomal degradation and are typically considered canonical ubiquitination signals. In contrast, K63-linked ubiquitination does not target proteins for proteasomal degradation but is instead involved in autophagy.

Thus, it is unclear why APT2 increases K48-linked ubiquitination while accompanied by a decrease in K63-linked ubiquitination. Further investigation is required to explain how TBK1 undergoes autophagic degradation despite the reduction in K63-linked ubiquitination.

- Compare TBK1 degradation in MG132-treated cells with Lactacystin or Epoxomicin (proteasome-specific inhibitors) to determine if degradation is proteasome

independent.

- *Generate TBK1 K48R or K63R mutants to analyze how each ubiquitination type affects TBK1 degradation and autophagy.*

Response: We appreciate the reviewer’s insightful comments and suggestions. To address the concern, we outline our responses as follows:

(1) **Ubiquitination Pathways:** We agree with the reviewer that K48-linked ubiquitination typically signals substrates for proteasomal degradation. However, *Liu et al* were the first to show that OPTN protein, when modified by K48 linked ubiquitin chains mediated by HACE1, is primarily degraded via a lysosome-dependent autophagy pathway¹ (**New Figure 3a**). Furthermore, several studies have indicated that under certain conditions, K48-linked ubiquitination can facilitate substrate degradation through the autophagy pathway, including the TBK1 protein²⁻⁴ (**New Figure 3b-g**). These findings illustrate the complexity of signals from ubiquitin chains, suggesting that they convey information through multiple pathways.

Editorial note: figure redacted Liu, Z. <i>et al. Cancer Cell</i> 26, 106-120 (2014).	Editorial note: figure redacted
Editorial note: figure redacted Jiao, Y. <i>et al. Autophagy</i> 20, 1762-1779 (2024).	Hu, Z. <i>et al. Sci Adv</i> 9, eadg2339 (2023).

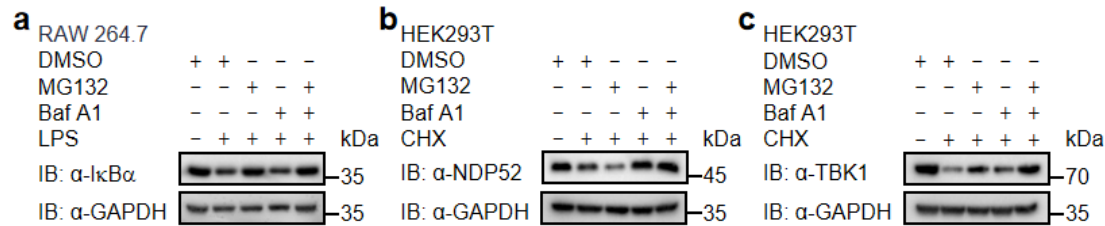
Editorial note: figure redacted

Hu, Z. *et al. Adv Sci (Weinh)* **11**, e2400952 (2024).

New Figure 3: The previous publications of the K48-linked ubiquitination for substrate degradation through autophagy. Not shown in the revised manuscript.

- (a) Fig. 2b in *Ref 1*, HACE1 mediates the K48 polyubiquitination ligation of OPTN to promote its degradation via the autophagy-lysosome pathway.
- (b,c) Fig. 5e and 7b in *Ref 2*, nsp2 mediates the K48 polyubiquitination ligation of TBK1 to promote its degradation via the autophagy-lysosome pathway.
- (d,e) Fig. 4g and 6a in *Ref 3*, VANG L2 mediates the K48 polyubiquitination ligation of TBK1 to promote its degradation via the autophagy-lysosome pathway.
- (f,g) Fig. 3f and 4b in *Ref 4*, GRA4 mediates the K48 polyubiquitination ligation of TBK1 to promote its degradation via the autophagy-lysosome pathway.

(2) **Degradation Pathways of TBK1:** To clarify TBK1's degradation pathway and validate the effectiveness of inhibitors, we monitored the degradation of typical substrates. Proteasome substrate I κ B α ⁸: In LPS-stimulated RAW 264.7 cells, the proteasome inhibitor MG132 blocked I κ B α degradation, while the autophagy inhibitor Baf A1 had no effect. Autophagy substrate NDP52⁹: In CHX-treated 293T cells, NDP52's degradation was blocked by Baf A1, while MG132 was ineffective. TBK1: In CHX-treated cells, both MG132 and Baf A1 partially reversed TBK1 degradation, and their combination completely prevented it (**New Figure 4**). These results indicate that TBK1 depends on both the proteasome and autophagy pathways for degradation, contrasting with the behavior of typical substrates of a single pathway (I κ B α , NDP52). Additionally, other key proteins such as ACE2, p65, cGAS, and MAVS have also been reported to be regulated by both the ubiquitin-proteasome system (UPS) and autophagy. Hence, TBK1's degradation pathway is context-dependent, influenced by various stimulation conditions and regulatory factors.

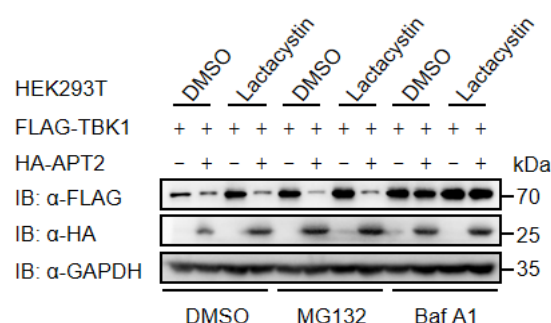


New Figure 4: Not shown in the revised manuscript.

(a) RAW 264.7 cells were treated with LPS (50 ng/mL) for 2 h, followed by treatment with MG132 (10 μ M), Baf A1 (0.2 μ M) or both for 6 h. Cell lysates were collected for immunoblot analysis.

(b,c) HEK293T cells were treated with cycloheximide (CHX) (100 mg/mL) for 24 h, followed by treatment with MG132 (10 μ M), Baf A1 (0.2 μ M) or both for 6 h. Cell lysates were collected for immunoblot analysis.

(3) APT2-Mediated Degradation of TBK1: To determine if APT2-mediated TBK1 degradation relies on the proteasome pathway, as suggested by the reviewer, we employed the more specific proteasome inhibitor Lactacystin. As shown in **New Figure 5**, Lactacystin effectively stabilized basal levels of endogenous TBK1. However, when APT2 was overexpressed, Lactacystin could not prevent TBK1 degradation. Even with a combination of proteasome inhibitors (MG132+Lactacystin), APT2 continue to degrade TBK1. In contrast, when we added autophagy-lysosome inhibitors (Baf A1+Lactacystin), Baf A1 completely restored APT2-mediated TBK1 degradation. These observations suggest that APT2-mediated degradation of TBK1 is dependent on the autophagy pathway and independent of the proteasome pathway. Relevant data have been added to the manuscript (Lines 278 - 283, Page 10).



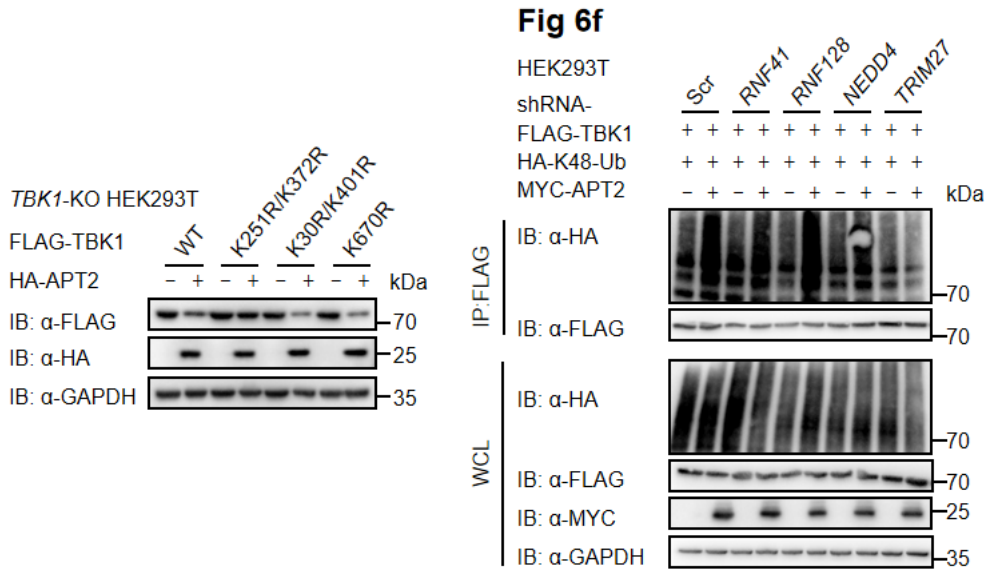
New Figure 5, presented as **Supplementary Fig. 5f** in the revised manuscript.

HEK293T cells were transfected with plasmids for FLAG-TBK1, together with HA-EV or HA-APT2 for 24 h. Then, cells were treated with DMSO (control) or Lactacystin (10 μ M) for 6 h, followed by treatment with DMSO (control), MG132 (10 μ M), or Baf A1 (0.2 μ M) for an additional 6 h. Cell lysates were collected for immunoblot analysis.

(4) Mechanism of APT2 Regulation: To elucidate how APT2 regulates TBK1 ubiquitination and degradation, we conducted two key experiments as following:

- The K251 and K372 are crucial sites for APT2-mediated K48 ubiquitination and degradation of TBK1:** After reviewing key literature^{7, 10, 11}, we recognize three sets of TBK1 lysine mutants related to K48 or K63 ubiquitin modifications: TBK1-K251R/K372R (K48 linkage), TBK1-K30R/K401R (K63 linkage), and TBK1-K670R (K48 linkage). Our results demonstrated that APT2 specifically induces K48-linked ubiquitination at K251 and K372 of TBK1, leading to autophagy-dependent degradation.

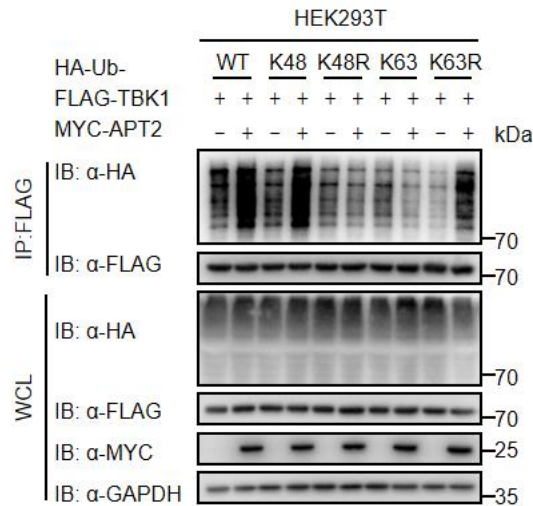
The K30/K401 sites and K670 are dispensable for APT2-mediated degradation (**New Figure 6**). Notably, while both TBK1-K251R/K372R and TBK1-K670R target known K48 ubiquitin, only K251/K372 double mutant resists APT2 degradation. This strongly suggests that APT2-mediated degradation is focused on a specific E3 ligase acting on K251/K372, not K670. Our study further demonstrates that APT2 recruits the specific E3 ligase TRIM27, rather than DTX4 (the previously reported E3 ligase associated with the K670 site), reinforcing TRIM27's central role in targeting K251/K372 (see **Fig. 6f**).



New Figure 6: Not shown in the revised manuscript.

TBK1-KO HEK293T cells were transfected with WT FLAG-TBK1 or its mutants, together with HA-EV or HA-APT2 for 24 h. Cell lysates were collected for immunoblot analysis.

- b) **APT2 Specifically Depends on K48 Ubiquitin Chains to Mediate TBK1 Modification:** To clarify the type of ubiquitin chain involved in APT2's action, we constructed ubiquitin point mutation plasmids (HA-Ub-K48R and HA-Ub-K63R). The experimental results showed that APT2 substantially enhances K48-linked polyubiquitination of TBK1. When the K48R mutant ubiquitin was present, this promoting effect of APT2 was completely abolished. Conversely, APT2 did not significant affect K63-linked ubiquitination, as it continue to enhance TBK1 ubiquitination in the presence of the K63R mutant ubiquitin. These findings confirm that APT2 drives the ubiquitination of TBK1 specifically through the catalysis of K48-linked polyubiquitination, rather than K63-linked modification. Relevant data have been added to the manuscript (**New Figure 7**, presented as **Fig. 6a**).



New Figure 7, presented as **Fig. 6a** in the revised manuscript.

HEK293T cells were transfected with FLAG-TBK1 and HA-tagged WT ubiquitin (HA-Ub) or its mutants, together with MYC-EV or MYC-APT2 for 24 h, followed by treatment with Baf A1 (0.2 μ M) for 6 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis.

3. Investigating the Role of TBK1 Palmitoylation in Ubiquitination During Plasmodium Infection

Fig. 1b and Fig. 7f show that the TBK1-IFN-I signaling pathway initially suppresses infection, but as Plasmodium immune evasion mechanisms activate, negative feedback leads to TBK1 degradation, ultimately increasing parasitemia again.

The authors propose that APT2 depalmitoylates TBK1, increases K48-linked ubiquitination, and promotes TBK1 degradation via autophagy. This raises the question of whether TBK1 palmitoylation suppresses K48-linked or K63-linked ubiquitination during the early stages of infection, before negative feedback is initiated. Thus, it is important to determine how TBK1 ubiquitination patterns (K48-linked, K63-linked) change when ZDHHC9 is overexpressed, thereby increasing TBK1 palmitoylation.

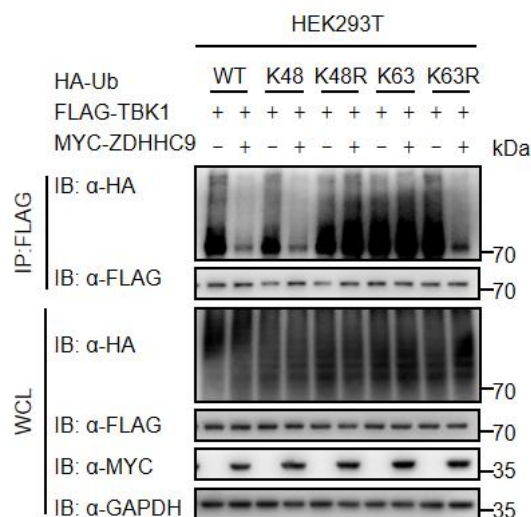
- *Investigate whether ZDHHC9 overexpression alters TBK1 ubiquitination patterns (K48/K63).*

- *Examine the temporal changes in TBK1 ubiquitination patterns during Plasmodium infection.*

Response: We sincerely appreciate the reviewers for highlighting this critical aspect. Below, we outline our responses:

(1) We agree with the reviewer that elucidating how palmitoylation affects the ubiquitin chain linkage type (K48/K63) of TBK1 is crucial for understanding its stability

mechanism. In line with the reviewer's suggestions, we performed experiments to explore the effects of ZDHHC9 overexpression on TBK1 ubiquitination patterns. Our findings indicate that ZDHHC9-mediated palmitoylation significantly reduces K48-linked polyubiquitination of TBK1 while not affecting K63-linked polyubiquitination. This suggests that palmitoylation helps maintain TBK1 stability by specifically inhibiting K48-linked polyubiquitination without regulating K63-linked ubiquitination. Relevant data have been added to the manuscript (**New Figure 8**, presented as **Supplementary Fig. 6b**) (Lines 320 - 323, Page 11).



New Figure 8, presented as **Supplementary Fig. 6b** in the revised manuscript.

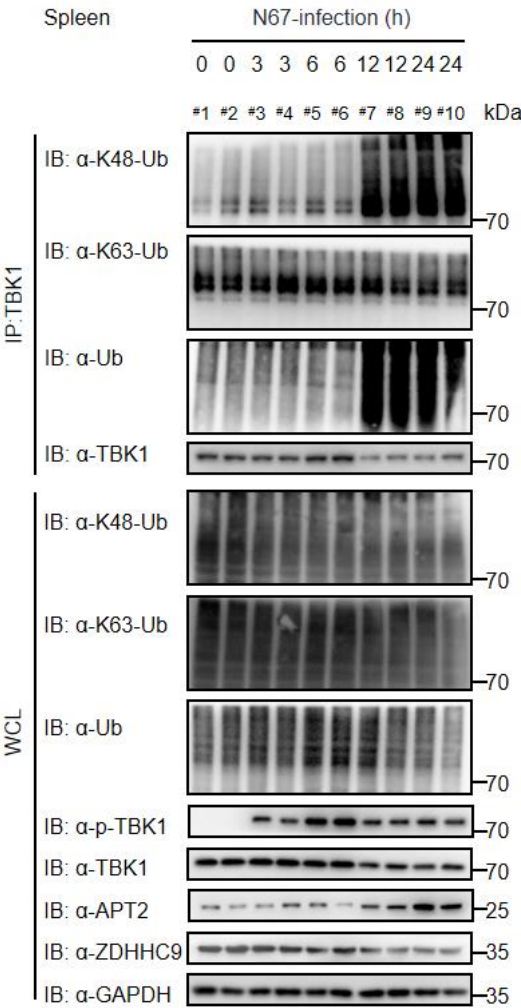
HEK293T cells were transfected with FLAG-TBK1 and HA-tagged WT ubiquitin (HA-Ub) or its mutants, together with MYC-EV or MYC-ZDHHC9 for 24 h, followed by treatment with Baf A1 (0.2 μ M) for 6 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis.

(2) To examine the temporal dynamics of TBK1 ubiquitination during *Plasmodium* infection, we conducted a time-course analysis in infected mice at 0, 3, 6, 12, and 24 h post-infection. The experimental results revealed the following (**New Figure 9**, presented as **Supplementary Fig. 6c**) (Lines 323 - 326, Page 11):

- Time-dependent shift in TBK1 ubiquitination patterns:** In the later stage of infection (12-24 h), we observed a significant increase in K48-linked polyubiquitination of endogenous TBK1, while K63-linked polyubiquitination remained stable. This demonstrates that K48-linked polyubiquitination is the primary mechanism driving TBK1 degradation in the later infection stages.
- Coordinated changes in the expression of regulatory enzymes:** During the same late infection stage (12-24 h), we detected that levels of the depalmitoylating enzyme APT2 were upregulated, while levels of the palmitoylating enzyme ZDHHC9 was downregulated. These changes corresponded with decreased levels of TBK1 and phosphorylated TBK1.
- Mechanistic integration:** The above results indicate that *Plasmodium* infection disrupts the balance between TBK1 palmitoylation and

depalmitoylation. We propose that the increased expression of APT2 drives TBK1 depalmitoylation, making it more susceptible to modification by K48-linked ubiquitin chains and leading to degradation via autophagy. This represents a core mechanism for regulating TBK1 homeostasis during the later stages of *Plasmodium* infection.

In summary, ZDHHC9 maintains TBK1 palmitoylation and inhibits K48-linked polyubiquitination during the early stages of infection (0-6 h). However, as the infection progresses to later stages (12-24 h), the downregulation of ZDHHC9 and upregulation of APT2 leads to TBK1 depalmitoylation. This process enhances K48-linked polyubiquitination, facilitating TBK1 degradation via the autophagy pathway and establishing a negative feedback regulatory loop.



New Figure 9, presented as **Supplementary Fig. 6c** in the revised manuscript. C57BL/6J mice were infected with N67 for various time points. Spleen tissue lysates were collected for IP (with anti-TBK1) and immunoblot analysis.

4. Evaluating the Impact of APT2 on IRF3 Phosphorylation and Nuclear Translocation

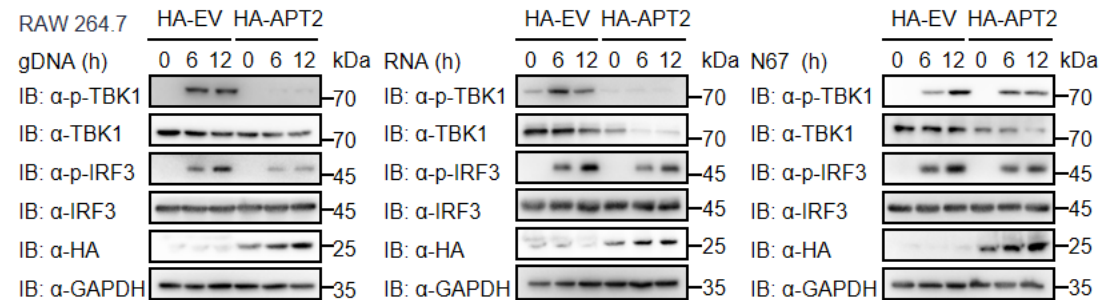
During Plasmodium Infection

The authors confirmed that *Plasmodium* infection leads to increased APT2 expression (Fig. 7a, b) and TBK1 instability, as demonstrated by the increased TBK1 degradation in CHX chase assays (Fig. 5e, f). Considering that APT2 expression increases during *Plasmodium* infection, and that TBK1 degradation should lead to reduced IRF3 phosphorylation or nuclear translocation, it is necessary to experimentally confirm whether APT2-mediated negative feedback reduces IRF3 phosphorylation and nuclear localization.

- Assess changes in IRF3 phosphorylation levels following APT2 overexpression.
- Examine IRF3 nuclear translocation following APT2 overexpression using confocal microscopy or nuclear fractionation.

Response: We appreciate the reviewer’s inquiries.

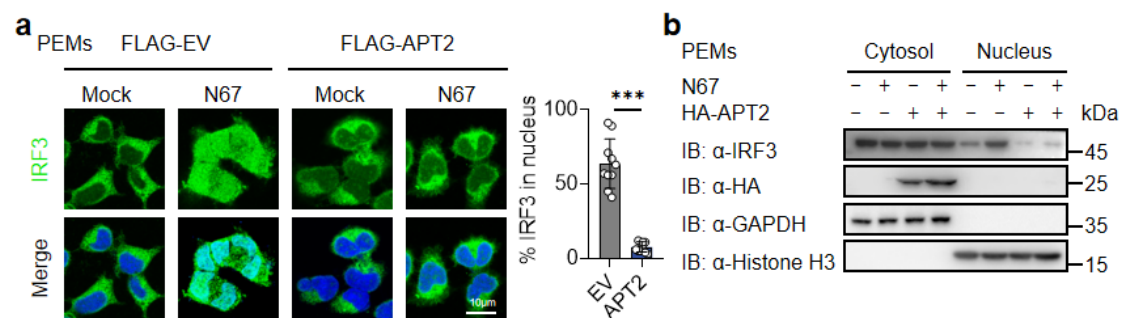
(1) In response to the first inquiry, we found that overexpression of APT2 led to a significant decrease in the levels of phosphorylated IRF3. This finding suggests that APT2 negatively regulates the phosphorylation and activation of IRF3 (**New Figure 10**, presented as **Fig. 4b**) (Line 226, Page 8).



New Figure 10, presented as **Fig. 4b** in the revised manuscript. RAW 264.7 cells were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with gDNA, RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for immunoblot analysis.

(2) To comprehensively investigate the role of APT2 in the nuclear translocation of IRF3, we conducted confocal microscopy and nuclear fractionation experiments on cells overexpressing APT2. Both methods consistently demonstrated that APT2 overexpression significantly inhibited IRF3 nuclear translocation when stimulated with freeze-thaw iRBCs (**New Figure 11**, presented as **Fig. 4c** and **Supplementary Fig. 4b**) (Lines 226-227, Page 8).

Collectively, these experimental results demonstrate that APT2-mediated degradation of TBK1 ultimately leads to reduced phosphorylation and nuclear translocation of IRF3.



New Figure 11, presented as **Fig. 4c** and **Supplementary Fig. 4b** in the revised manuscript.

(a) PEMs were transfected with FLAG-EV or FLAG-APT2 for 24 h, then stimulated with freeze-thawed iRBCs for 12 h. IRF3 (green) and nucleus (DAPI, blue) was examined by confocal microscopy. Scale, 10 μ m. Quantitative analyses of the colocalization (10 cells per group) are presented next to the image.

(b) Cellular fractionation and immunoblot analysis of IRF3 in PEMs were transfected with HA-EV or HA-APT2 for 24 h, then stimulated with freeze-thawed iRBCs for 12 h. Data with error bars are shown as mean $\pm$ SD. Each panel is a representative experiment of at least three independent biological replicates. *** P < 0.001 as determined by unpaired Student's t test.

Minor

5. The legend for the 'Uninfected group' is missing in Fig. 1g.

Response: We apologize for this oversight regarding the 'Uninfected group' legend in Fig. 1g. This has been corrected in the revised manuscript. Thank you for bringing it to our attention.

Responses to Reviewer #2:

Reviewer #2:

The manuscript by Han et al. clearly demonstrates that TBK1 palmitoylation is involved in type-1 interferon production in models of malaria infection. This is antagonized by APT2, which depalmitoylates TBK1 leading to its degradation via autophagy. This is a very strong manuscript that could interest experts in immune responses against parasitic infections. However, a few additional experiments would broaden the scope of this manuscript, making it more relevant to the field of innate immunity.

Response: We thank the reviewer very much for the insightful and thorough comments.

Major points:

1. TBK1 is also an important kinase in TRIF-mediated signaling (Fitzgerald et al., Nature Immunology, 2003), a signaling pathway that is activated upon exposure to Plasmodium (Couper et al., Plos Pathogens, 2010) (Zhang et al., Exp Parasitology, 2016). So, it is possible that the observed effects are TLR-mediated, not only STING/MAVS as suggested. Further experimentation to establish which pathway(s) is involved in TBK1 palmitoylation/depalmitoylation would give us a clearer understanding of the signaling events involved.

Response: We appreciate the reviewer's insightful comment regarding the potential role of the TLR-TRIF pathway in regulating TBK1 palmitoylation, which prompted us to explore the contribution of TRIF to TBK1 palmitoylation/depalmitoylation.

To investigate this, we performed siRNA knockdown of *Ticam1* (encoding TRIF), *Mavs*, or *Tmem173* (encoding STING). Our results indicate that the knockdown of any of these three molecules (**New Figure 12a**) led to a diminished reduction in TBK1 palmitoylation when induced by freeze-thawed N67 iRBCs (**New Figure 12b**). This suggests that both the STING/MAVS and TLR-TRIF pathways are crucial for mediating N67-induced TBK1 depalmitoylation, potentially functioning through redundancy or interdependent crosstalk.

Additionally, we examined the expression levels of key palmitoyltransferases and acyl protein thioesterases, specifically ZDHHC9 and APT2. Our findings revealed that N67 upregulated APT2 expression while downregulating ZDHHC9 expression. Notably, the knockdown of STING, MAVS, or TRIF partially reversed the N67-induced alterations in APT2 and ZDHHC9 expression. This establishes a clear connection between STING/MAVS, and TRIF-dependent signaling pathways and the transcriptional regulation of enzymes that modulate TBK1 palmitoylation (**New Figure 12c**).

In conclusion, our data support a model in which the *Plasmodium* activates multiple innate immune sensing pathways, including STING/MAVS and TLR-TRIF. These pathways converge to remodel the expression of palmitoylation regulatory

enzymes, ultimately resulting in TBK1 depalmitoylation. Relevant data have been added to the manuscript (Discussion, Lines 466-472).

[editorial note: figure redacted due to its containing unpublished data]

2. The manuscript focused on Plasmodium parasites and established clear links between TBK1 palmitoylation/depalmitoylation with ZDHHC9/APT2 in this context. Are these players the same in other contexts (either other infections or in cells stimulated with “clean” agonists)?

Response: We thank the reviewer for raising the essential question regarding the applicability of the TBK1 palmitoylation/depalmitoylation mechanism mediated by ZDHHC9/APT2 in the contexts beyond *Plasmodium* infection. To address this, we conducted experiments using a variety of stimuli, including infections with VSV, HSV, *Toxoplasma gondii* (*T. gondii*), and stimulations with various agonists, including LPS, poly(I:C), and poly(dA:dT). Our key findings demonstrate:

(1) **Shared Phenotype (Depalmitoylation of TBK1):** In line with our observations during *Plasmodium* infection, we discovered that *T. gondii* infection, as well as stimulation with poly(I:C) or poly(dA:dT), significantly impaired the palmitoylation of TBK1 (**New Figure 13a**). This indicates that TBK1 depalmitoylation is a common response across various immune challenges, particularly those associated with nucleic acid sensing.

(2) **Stimulus-Specific Regulation of Enzymes:** The regulation of enzymes APT2 and ZDHHC9 exhibited notable stimulus-specific patterns. For instance, poly(I:C) stimulation led to a significant upregulation of both depalmitoylases APT1 and APT2, while several palmitoyltransferases in the ZDHHC family-including ZDHHC9 as well as ZDHHC1, ZDHHC5, and ZDHHC8-were downregulated (**New Figure 13b**). In contrast, *T. gondii* infection and poly(dA:dT) stimulation primarily led to the upregulation of PPT2 and downregulation of other palmitoyltransferases, excluding ZDHHC9 (**New Figure 13b**).

(3) **Other infections/stimulation:** Infections with VSV or HSV, as well as LPS stimulation, did not significantly alter TBK1 palmitoylation levels (**New Figure 13a**).

[editorial note: figure redacted due to its containing unpublished data]

These results demonstrate that while TBK1 depalmitoylation represents a shared response to various nucleic acid-sensing stimuli, the regulatory mechanisms governing the ZDHHC9/APT2 enzyme pair are highly context-dependent. Our findings confirm the core ZDHHC9/APT2 axis identified in the *Plasmodium* model but highlight significant variations in upstream regulation based on the specific stimulus. We appreciate the reviewer's emphasis to this point, which represents a critical area of our ongoing research. We plan to explore these aspects in more detail and publish the findings in future work. Therefore, a comprehensive discussion of this topic has been omitted from the current manuscript.

3. Figure 2k is not very clear. ZDHHC9 has a FLAG-tag, but what about TBK1? Additionally, HEK expression systems can lead to high expression levels, forcing interactions that don't necessarily occur at physiological levels. A control using an unrelated protein would strengthen this figure.

Response: We are grateful to the reviewers for their valuable comments, which have significantly improved the rigor of our research. In addressing the concerns regarding Figure 2k, we have made the following clarifications and additions:

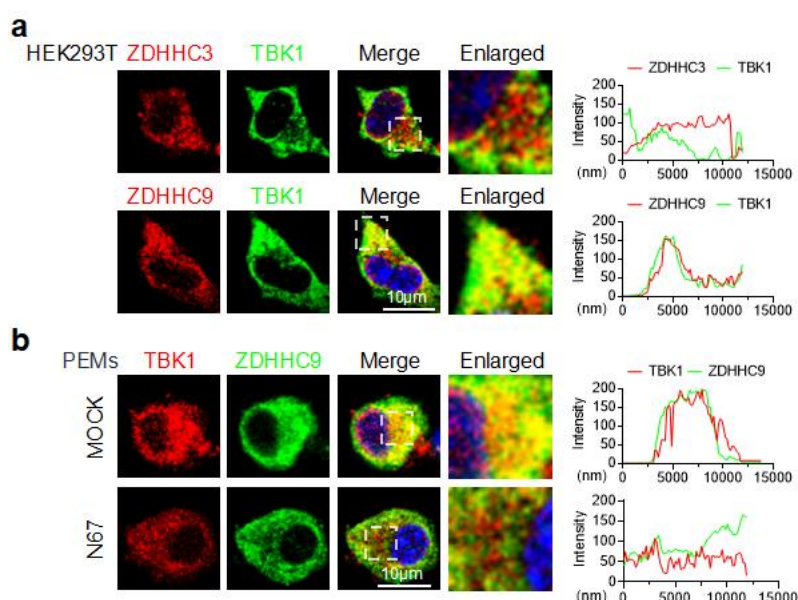
(1) **Clarification on TBK1 Detection:** In Figure 2k, we utilized antibodies against endogenous TBK1 (which is untagged) for detection. In contrast, ZDHHC9 was detected through its FLAG tag following overexpression.

(2) **Introduction of Specificity Controls:** We agree with the reviewer that overexpression in the HEK293T system could lead to non-physiological interactions. To validate the specificity of the ZDHHC9-TBK1 interaction and mitigate potential false positives from overexpression, we performed control experiments with an unrelated protein. We co-expressed endogenous TBK1 alongside FLAG-ZDHHC9 (or the control protein FLAG-ZDHHC3) in HEK293T cells. Confocal microscopy analysis revealed significant co-localization signals for FLAG-ZDHHC9 and endogenous TBK1, whereas no detectable co-localization was observed for FLAG-ZDHHC3, which belongs to the same enzyme family (**New Figure 14a**). This negative control strongly supports the specificity of the ZDHHC9-TBK1 interaction.

(3) **Validation of Endogenous Interaction to Confirm Physiological Relevance:** To further confirm the ZDHHC9-TBK1 interaction under physiological conditions, we

performed confocal microscopy analysis in PEMs using antibodies against endogenous ZDHHC9 and TBK1. The results demonstrated significant co-localization between endogenous ZDHHC9 and endogenous TBK1 under basal conditions, with this co-localization being notably reduced following stimulation with freeze-thawed iRBCs (**New Figure 14b**). This endogenous experiment reinforces the physiological relevance of the ZDHHC9-TBK1 interaction.

In summary, we have enhanced the reliability and physiological relevance of Figure 2k by: (1) clarifying the detection method for TBK1, (2) introducing a negative control (ZDHHC3) to validate specificity in the overexpression system, and (3) confirming the endogenous interaction and its dynamic regulation in PEMs. Relevant data have been added to the manuscript (Lines 163-167, Page 6).



New Figure 14, presented as **Fig. 2m** and **Supplementary Fig. 2f** in the revised manuscript.

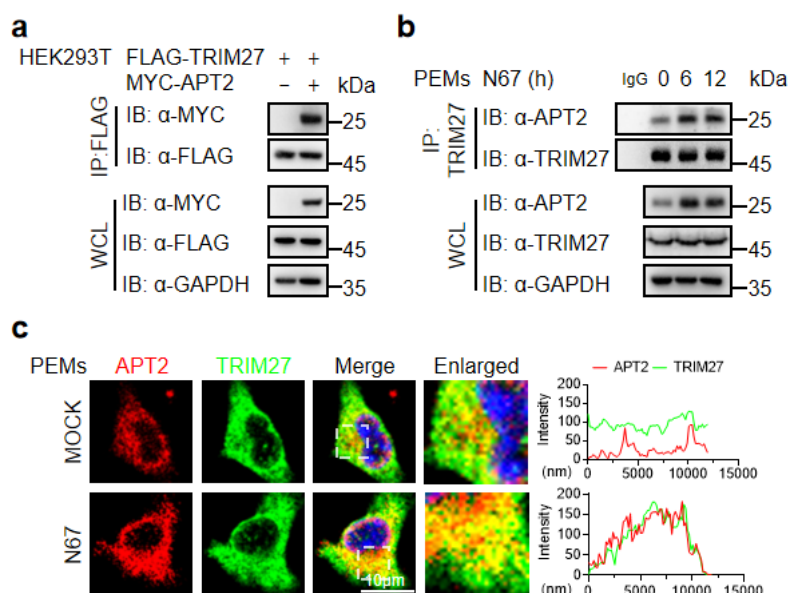
(a) HEK293T cells were transfected with FLAG-ZDHHC3 or FLAG-ZDHHC9. The colocalization between FLAG-ZDHHC3/9 (red) and TBK1 (green) was examined by confocal microscopy. The nucleus was stained with DAPI (blue). Scale, 10 μm. The intensity analysis is next to the image. The interaction between FLAG-ZDHHC3 and TBK1 was used as a negative control.

(b) PEMs were stimulated with freeze-thawed iRBCs for 12 h. The colocalization between TBK1 (red) and ZDHHC9 (green) was examined by confocal microscopy. The nucleus was stained with DAPI (blue). Scale, 10 μm. The intensity analysis is next to the image.

4. Is there any direct evidence that APT2 and TRIM21 physically interact? (Co-IP, colocalization, etc?)

Response: We appreciate the reviewer's insightful comments. It appears there was a misunderstanding, as our study focuses on TRIM27 rather than TRIM21. We interpret this query as concerning the potential interaction between APT2 and TRIM27.

Although our original manuscript did not explore the interaction between APT2 and TRIM27, we have conducted key experiments in response to reviewer's suggestions. Our results from both exogenous and endogenous Co-IP experiments confirm the specific binding of APT2 to TRIM27 (**New Figure 15a,b**). Furthermore, confocal microscopy analysis illustrates a clear colocalization of APT2 and TRIM27 (**New Figure 15c**). Notably, stimulation with freeze-thawed iRBCs significantly enhances this interaction (as evidenced by Co-IP) and increases the degree of colocalization (as evidenced by confocal) (**New Figure 15b,c**). Relevant data have been added to the manuscript (Lines 352-355, Page 12).



New Figure 15, presented as **Fig. 6i,j** and **Supplementary Fig.6i** in the revised manuscript.

(a) HEK293T cells were transfected with FLAG-TRIM27 and MYC-EV, -APT2 for 24 h. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis.

(b) PEMs were stimulated with freeze-thawed iRBCs at the indicated time points. Cell lysates were collected for IP and immunoblot analysis.

(c) PEMs were stimulated with freeze-thawed iRBCs for 12 h. The colocalization between APT2 (red) and TRIM27 (green) was examined by confocal microscopy. The nucleus was stained with DAPI (blue). Scale, 10 μm. The intensity analysis is next to the image.

Minor points:

5. (Introduction): *There isn't a clear consensus on whether hemozoin itself activates pattern recognition receptors, as it is likely that hemozoin needs to be "contaminated"*

with other molecules to stimulate PRRs (Parroche et al, PNAS, 2007).

Response: We appreciate the reviewer's insightful comments. As suggested, we have revised the introduction to reflect this point (Introduction, Lines 44-45).

6. (Introduction): In addition to STING/MAVS, TBK1 is also activated via TRIF (Fitzgerald et al., Nature Immunology, 2003).

Response: We thank the reviewer for this valuable feedback. We have included a clear acknowledgment in the revised manuscript (Introduction, Lines 55-58) that TBK1 is also activated via the TRIF pathway in addition to the STING/MAVS pathway. The related references have also been properly cited.

7. (Introduction): NF- κ B and IRF3/7 are transcription factors activated by various signaling pathways such as TLRs, IL-1Rs, cGAS/STING, etc. I believe that saying that NF- κ B and IRF3/7 are signaling pathways is conceptually inaccurate.

Response: We are grateful to the reviewer for their thorough review and for pointing out the conceptual imprecision in describing NF- κ B and IRF3/7 as signaling pathways. We recognize that this characterization was incorrect and have revised the relevant text in the manuscript to address this issue (Introduction, Lines 45-47).

8. (Results): (Figure 1) TBK1 phosphorylation goes down, but so does the total protein levels. Are there differences in the p-TBK1/TBK1 ratio.

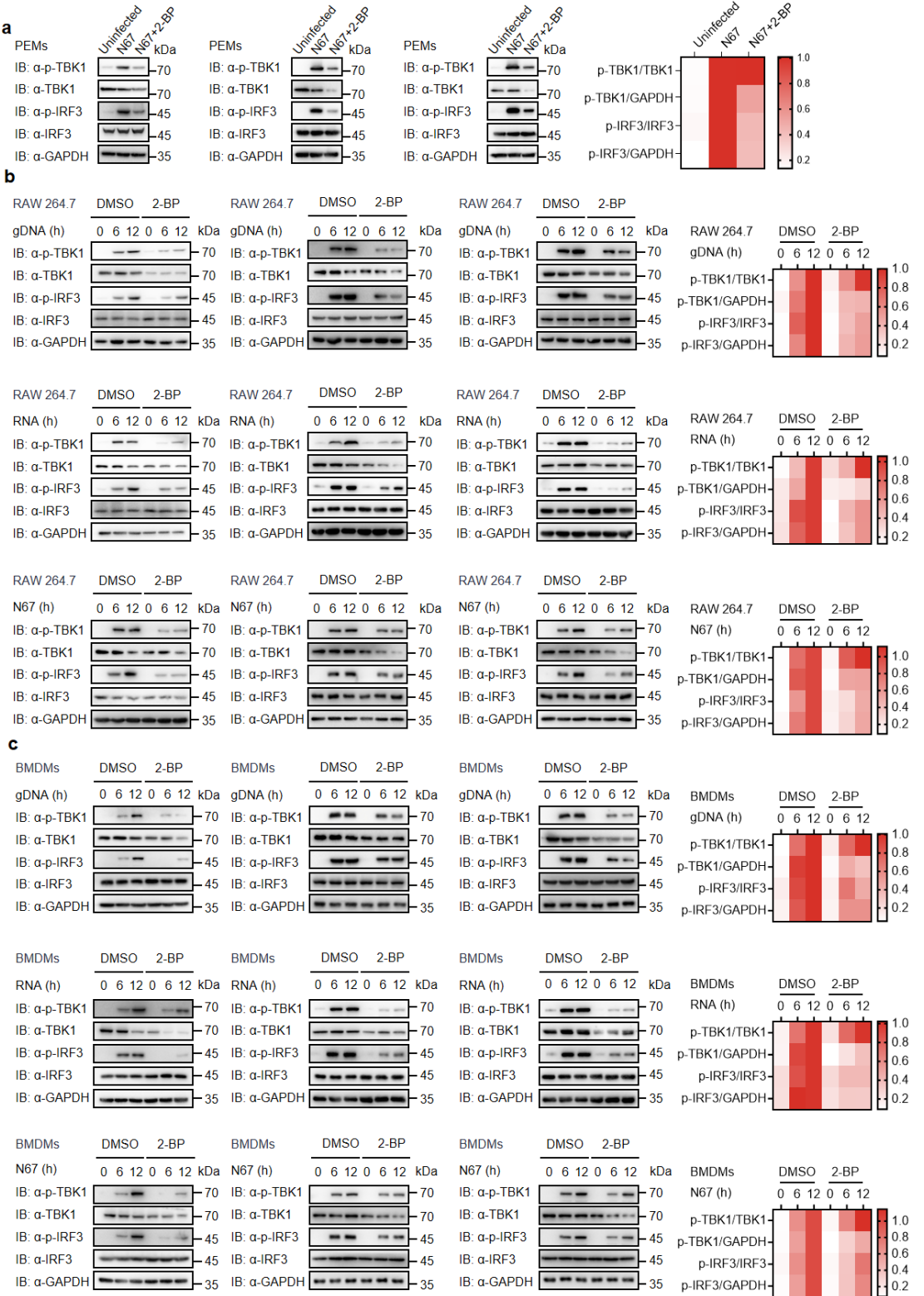
Response: We appreciate the reviewer for highlighting the changes in TBK1 phosphorylation (p-TBK1) and total TBK1 protein levels presented in Figure 1.

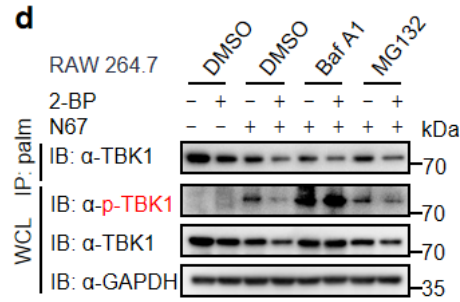
(1) As noted, treatment with 2-BP significantly reduced both p-TBK1 and total TBK1 levels. An analysis of the p-TBK1/TBK1 ratio showed no significant change following 2-BP treatment (**New Figure 16 a to c**). This is due to the 2-BP-induced depalmitoylation of TBK1, which promotes its degradation. The reduction in total TBK1 levels consequently leads to a corresponding decline in its phosphorylated form. Therefore, while both forms of the protein decrease in absolute terms, the ratio of p-TBK1/TBK1 remains unchanged.

(2) Additionally, when we treated cells with autophagy inhibitor Baf A1 to stabilize total TBK1, we observed a rescue in TBK1 phosphorylation levels (**New Figure 16d**), further supporting that the decrease in p-TBK1 is a result of total TBK1 degradation.

(3) Although the p-TBK1/TBK1 ratio did not change after 2-BP treatment, it is important to note that 2-BP treatment did suppress IFN-I signaling. Specifically, ratios of p-TBK1/GAPDH and the downstream transcriptional factor p-IRF3/IRF3 and p-IRF3/GAPDH dramatically decreased following 2-BP treatment (**New Figure 16 a**

to c). Collectively, these findings demonstrate that 2-BP inhibits IFN-I signaling by depalmitoylating and destabilizing TBK1, which in turn hinders its phosphorylation and downstream signaling activation.





New Figure 16 a to d: 16a and 16d presented as **Fig. 1h** and **Supplementary Fig. 5d** in the revised manuscript. Other representative heatmaps were not shown in the revised manuscript.

(a) C57BL/6J mice were injected *i.p.* with DMSO or 2-BP (40 mg/kg) for 4 days, followed by an injection of N67 iRBCs (1×10^6 , *i.p.*) for 24 h. Immunoblotting was conducted to assess the levels of total IRF3 and TBK1, as well as phosphorylated IRF3 and TBK1, on day 1 after N67 infection in PEMs. Representative heatmaps display the results of three independent replicate experiments. The grayscale values from each replicate were quantified, and the average value across the three replicates was calculated. This average was then normalized using the group with the highest grayscale value as the baseline (set to 1), to generate the final heatmap.

(b,c) RAW 264.7 cells (b) or BMDMs (c) were pre-treated with DMSO or 2-BP (100 mM for 24 h) and then stimulated with gDNA, RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for immunoblot analysis. Representative heatmaps display the results of three independent replicate experiments. The grayscale values from each replicate were quantified, and the average value across the three replicates was calculated. This average was then normalized using the group with the highest grayscale value as the baseline (set to 1), to generate the final heatmap.

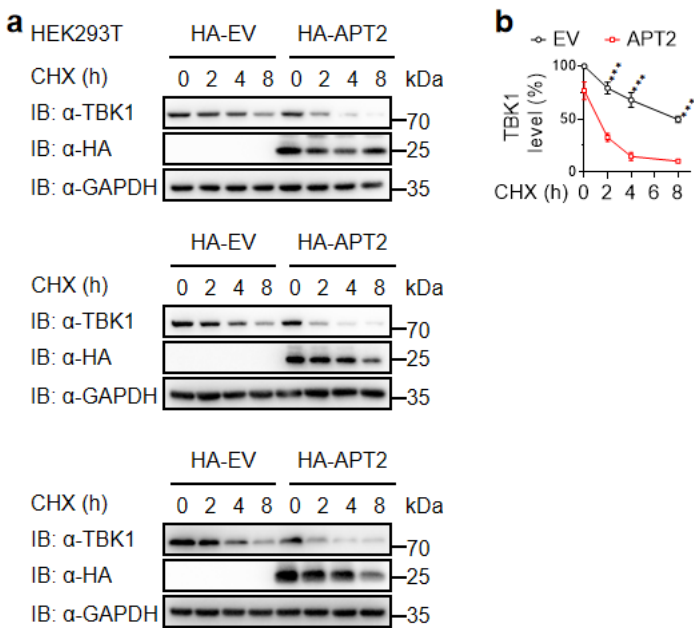
(d) RAW 264.7 cells were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with freeze-thawed iRBCs for 12 h and then DMSO (control), Baf A1 (0.2 μ M), or MG132 (10 μ M) treatment for 6 h. Cell lysates were collected for ABE assay and immunoblot analysis.

9. (Results): *“Furthermore, Co-IP experiments showed that APT2 physiologically interacts with TBK1 (Supplementary Fig. 3e)”. This refers to an experiment using HEK cells transfected with various proteins, it is not accurate to call this a physiological condition.*

Response: We sincerely thank the reviewer for raising this critical point, which prompted us to clarify our data more rigorously. We agree that conducting Co-IP experiments using HEK cells does not constitute evidence of interaction under physiological conditions, and have revised the manuscript accordingly to reflect this clarification (Lines 204-208, Page 7).

10. (Results): Differences in Figure 5g are not visually obvious. Densitometric quantification might make this point a bit more convincing.

Response: We thank the reviewer for this valuable suggestion. To address this point quantitatively and objectively, we have conducted densitometric quantification of the protein bands in Figure 5g using three independent biological replicates. Statistical analysis via an unpaired Student's *t*-test confirmed that the observed differences are indeed highly significant. We hope that this quantitative data strengthens the robustness of our conclusion regarding the expression differences. These results are now presented in the revised manuscript (**New Figure 17**, presented as **Fig. 5g,h**).



New Figure 17, presented as **Fig. 5g,h** in the revised manuscript.
(a,b) HEK293T cells were transfected with HA-EV or HA-APT2 for 24 h, then treated with CHX (100 mg/mL) at the indicated time points. Cell lysates were collected for immunoblot analysis (a). Quantitative analyses (b) of TBK1 signals.

11. (Methods): Part of “Western blotting and Co-immunoprecipitation” needs to be rewritten.

Response: We thank the reviewer for highlighting the need for improvements in the descriptions of the Western blotting and Co-immunoprecipitation methods. In response to this feedback, we have thoroughly rewritten these sections to provide more comprehensive and detailed protocols. The revised methods have been incorporated into the updated manuscript (Lines 552-574, Page 18).

Responses to Reviewer #3:

Reviewer #3:

The manuscript entitled “Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity” presents an in-depth study on the palmitoylation of the TBK1 protein and its impact on malaria immunity. While the study is interesting and potentially impactful, there are several serious concerns that need to be clarified before the manuscript can be considered for publication.

Response: We sincerely thank the reviewer for their insightful comments on our manuscript and are pleased to address all concerns raised.

Major points:

1. What is the rationale for focusing on type-I interferons in the context of malaria infection? The manuscript does not elaborate on why this specific pathway was chosen over others, despite the fact that numerous immune signaling pathways are dysregulated during malaria infection. Since type-I interferons are more commonly associated with antiviral responses, the study should explain their relevance in malaria pathogenesis. Including supporting data or references that highlight the role of type-I interferons in Plasmodium infections would strengthen the justification for this focus.

Response: We appreciate the opportunity to elaborate on our rationale for focusing on type I interferons (IFN-I) in the context of malaria infection.

(1) **Significance of IFN-I in *Plasmodium* Infections:** Research has extensively documented the role of IFN-I signaling pathways in malaria. Specifically, numerous studies indicate that IFN- α/β cytokines are vital for promoting protective host immune responses against *Plasmodium* (Supporting data are summarized in New Figure 18):

- a) During the liver stage of malaria infection, liver cells recognize *Plasmodium* RNA, activating IFN-I responses via the MDA5-IRF3 pathway. This mechanism serves as a crucial immune defense against hepatic malaria infection. Notably, *Ifnar*^{-/-} mice demonstrated a significantly higher liver parasite burden compared to WT mice¹³ (**New Figure 18a**).
- b) Blood-stage *Plasmodium* infection suppresses liver-stage development through IFN-I-mediated inhibition of hepatic replication¹⁴ (**New Figure 18b**).
- c) In blood-stage infections with *Plasmodium yoelii* (*P. yoelii*) strains, an early surge in IFN-I (within 18-24 h) correlates with reduced parasite growth and improved host survival; furthermore, the magnitude of this initial IFN-I response determines its protective efficacy¹⁵ (**New Figure 18c,d**).
- d) In early *P. yoelii* 17XL infections, activation of the TLR7-IRF7 pathway induces IFN- α/β production, promotes T-cell differentiation, enhances anti-malarial immunity, and thereby reduces parasitemia¹⁶ (**New Figure**

18e,f).

- e) In mice infected with *P. berghei* ANKA (*PbANKA*), daily IFN- α administration initiated at infection significantly reduced the incidence of cerebral malaria¹⁷

(New Figure 18g,h).

Collectively, these findings underscore the protective role of IFN-I throughout the malaria infection cycle, highlighting its mechanisms of action against liver-stage parasites, suppression of blood-stage growth, enhancement of anti-malarial immunity, and reduction of severe complications such as cerebral malaria.

(2) **Relevance of Our data:** Our study, through RNA-seq analysis (Fig. 1d-f), identified that palmitoylation predominantly regulates the IFN-I signaling pathway during *Plasmodium* N67 infection. Given the central importance of IFN-I in immune defense against malaria and our findings regarding its regulation by palmitoylation, it is critical to further investigate the functional and regulatory mechanisms of IFN-I in the malaria infection model.

(3) **Complex Role of IFN-I:** It is important to note that the role of IFN-I is multifaceted and can act as a “double-edged sword” in different infections and diseases. While it provides crucial protective effects against most pathogens, it may also contribute to immunopathological damage or exacerbate disease progression in case of chronic/latent infection or tumor immune escape¹⁵⁻²⁶. This duality emphasizes the need for in-depth exploration of the specific mechanisms and regulatory networks associated with IFN-I.

In response to your suggestion, we have incorporated relevant contents in the introduction to strengthen the justification for our focus on type I interferon signaling of this study (Line 51-54, Page 3).

Editorial note: figure redacted

Liehl, P. *et al. Nat Med* **20**, 47-53 (2014).

Editorial note: figure redacted

Patel, H. *et al. Nat Commun* **15**, 2104 (2024).

Editorial note: figure redacted

Yu, X. *et al. Immunity* **45**, 1093-1107 (2016).

Editorial note: figure redacted

Lu, J. *et al. J Adv Res* **73**, 295-310 (2025).

Editorial note: figure redacted

Vigário, A.M. *et al. J Immunol* **178**, 6416-6425 (2007).

New Figure 18, relevant literature on the host protective effects of IFN-I in anti-*Plasmodium* immune responses. Not shown in the revised manuscript.

(a) Fig. 3a in Ref 13, parasite liver load in WT and *Ifnar*^{-/-} mice measured by qRT-PCR of *P. berghei* 18S rRNA, 42 h and 48 h after infection normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and plotted as percentage of the levels in WT mice.

(b) Fig. 6e in Ref 14, anti-IFN γ /anti-IFNAR1 (n=6 mice per group) monoclonal antibodies. Four days later, the mice were infected with 50,000 *Pb* ANKA^{luc} sporozoites. Parasite liver stage burden was measured by IVIS at 42-43 hpi and represented as total flux (p/s).

(c,d) Fig. 2a in Ref 15, parasitemias and mortality rates of WT mice infected with YM, followed by *i.v.* administration of recombinant mouse IFN- α/β , IL-6, IFN- γ , or control BSA protein at 18 h post infection.

(e,f) Fig. 2a and 2b in Ref 16, parasitemias (e) and mortality rates (f) of WT and *Ifnar*^{-/-} mice infected with 17XL (0.5×10^6 iRBCs) (n $\geq$ 4).

(g,h) Fig. 1 in Ref 17, effect of IFN- α treatment on survival and parasitemia. Survival (g) and parasitemia (h) of mice infected with 10^6 PbA iRBC and treated with 4×10^5 mouse units of IFN- α from days 0 to 25 (g) or from days 3 to 12 (h). Control mice were

infected with PbA and received only diluent.

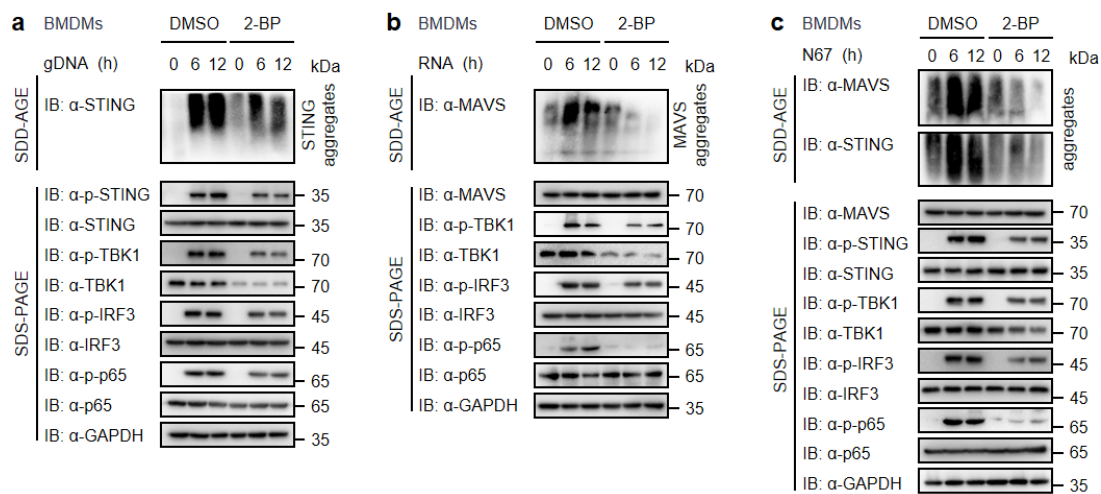
2. The authors should clarify why only TBK1 and IRF3 were examined in the Western blot analyses. Given that both STING and MAVS function upstream of TBK1, it would be important to assess their involvement to better understand the signaling cascade. Additionally, since TBK1 also regulates the NF- κ B pathway, examining key NF- κ B markers such as phosphorylated I κ B α or p65 would provide a more comprehensive view of TBK1's role in the immune response to malaria infection. Expanding the analysis to include these upstream and downstream components would strengthen the study's mechanistic insights.

Response: We sincerely thank the reviewers for their insightful comments and suggestions, which have greatly improved our study. In response, we have conducted additional experiments to comprehensively evaluate the roles of STING, MAVS, and NF- κ B signaling in the immune response to malaria infection. Our new data demonstrate the following:

(1) **Analysis of STING and MAVS:** We found that treatment with 2-BP significantly inhibits the phosphorylation of STING and oligomerization of MAVS following N67 stimulation. Importantly, our data indicate that 2-BP directly destabilizes the TBK1 protein, which in turn inhibits its downstream signaling activation. This regulatory mechanism affecting TBK1 stability operates independently of the inhibitory effects of 2-BP on the activity of STING and MAVS, which did not influence their protein stability. This discovery represents a novel mechanism by which 2-BP inhibits IFN-I production. These findings are illustrated in **New Figure 19** and further discussion about palmitoylation of TBK1 regulated anti-malaria infection is independent on its regulation of STING and MAVS is included in response to question # 8 by Reviewer 3.

(2) **Investigation of NF- κ B Signaling:** Beyond mediating IRF3/7 activation, TBK1 is also known to facilitate the NF- κ B signaling pathway by directly phosphorylating the p65 subunit of NF- κ B. Our experiments demonstrated that 2-BP treatment results in a significant decrease in the phosphorylation levels of p65, confirming its inhibition on N67 infection mediated NF- κ B activation (**New Figure 19**).

These results from **New Figure 19** elucidate 2-BP treatment disrupts the entire signaling cascade—from upstream adaptor proteins (STING/MAVS) to the stability of the kinase TBK1, and finally to downstream activation of both IRF3 and NF- κ B. This comprehensive mechanistic analysis robustly supports our conclusion that 2-BP broadly modulates host immune responses during malaria infection by targeting multiple key nodes within the immune signaling cascade. We sincerely thank the reviewers for their highly constructive and valuable suggestion, which has significantly enhanced the depth and scope of this study. The relevant results have been clearly incorporated into the revised manuscript (Lines 115-118, Page 5).



New Figure 19, presented as **Fig. 1i-k** in the revised manuscript.

(a-c) BMDMs were pre-treated with DMSO or 2-BP (100 mM for 24 h), then stimulated with gDNA, RNA or freeze-thawed iRBCs at indicated time points. Cell lysates were collected for immunoblot analysis.

3. The authors should provide a more detailed explanation of the infection model used in their experiments. In the study, PBS was used as a control, while iRBCs were used for infection. However, it is important to clarify how this model accurately mimics natural infection conditions and ensures that the observed immune response is due to the infection itself rather than simply the presence of foreign nucleic acids. Was the parasite live in iRBCs? Additional controls, such as uninfected RBCs or heat-killed iRBCs, could help distinguish between infection-specific immune activation and a general response to foreign nucleic acids.

Response: We sincerely thank the reviewers for raising these highly important points regarding our infection model and the specificity of immune activation. We agree that it is of crucial to accurately distinguish between infection-specific responses and general responses to foreign components. Below is our detailed response:

(1) Malaria Parasite Modeling: The malaria modeling utilized in our study involved infected red blood cells (iRBCs), a method widely validated by existing research²⁷⁻²⁹. The parasites within the iRBCs used in this study were maintained in a viable and metabolically active state. Prior to each experiment, parasite viability was assessed under standardized culture conditions (**New Figure 20a,b**).

(2) Control Experiments to Address Immune Activation Specificity: We acknowledge the reviewer's concerns regarding the specificity of immune activation. To address this, we conducted critical control experiments (**New Figure 20c,d,f**):

a) **Uninfected RBCs:** Our use of uninfected RBCs as a control demonstrated

that they failed to induce any detectable IFN-I response or trigger the key molecular changes observed during *in vivo* iRBC infection.

- b) **Heat-killed iRBCs:** We also included heat-killed iRBCs to discern the role of live parasites in our model. This treatment effectively abolished parasite viability while preserving the overall cellular structure and nucleic acid components. Our experimental data show that heat-killed iRBCs could not activate the IFN-I response.
- c) Collectively, these two control experiments provide compelling evidence that neither uninfected RBCs nor the nucleic acids present in heat-killed iRBCs are sufficient to trigger the IFN-I signaling pathway. Thus, the immune activation observed in our experiments depends primarily on the presence of viable, replicating *Plasmodium* parasites within the iRBCs.

(3) **Analysis of ZDHHC9-APT2 Axis:** To further establish the infection specificity of the downstream molecular response, we analyzed key enzymes involved in TBK1 palmitoylation under identical control conditions. Exposure to iRBCs significantly downregulated both mRNA and protein levels of ZDHHC9, while concurrently upregulating APT2 mRNA and protein levels. Crucially, neither uninfected RBCs nor heat-killed iRBCs induced these alterations. Consistent with disruption of the ZDHHC9-APT2 axis, a reduction in TBK1 palmitoylation was observed only in samples treated with iRBCs. This post-translational modification remained unchanged upon exposure to uninfected RBCs or heat-killed iRBCs, reinforcing that the observed molecular signature is strictly dependent on active infection (**New Figure 20e,f**).

In conclusion, these experimental results strongly demonstrate that, compared to the robust immune response induced by infected RBCs, both uninfected RBCs and heat-killed iRBCs fail to elicit an IFN-I response. This indicates that our model effectively trigger a specific immune response against active *Plasmodium* infection. Importantly, this immune activation does not arise merely from the presence of foreign red blood cells or nucleic acids, but rather requires the presence of live, replicating parasites within iRBCs. Thus, our model closely mimics a critical aspect of the natural infection process.

[editorial note: figure redacted due to its containing unpublished data]

4. Another important consideration is whether the experiments with N67 gDNA and RNA included appropriate control DNA or RNA. This is crucial because nucleic acids, in general, can stimulate immune responses. Without proper controls it would be difficult to determine whether the observed immune activation was specific to malaria or simply a general response to foreign nucleic acids. Were such controls included in the experiments?

Response: We appreciate the reviewer's insightful comments regarding the potential for non-specific immune activation due to the use of N67 gDNA and RNA. We would like to clarify the comprehensive nature of our experimental design and the control measures we employed to confirm the specificity of our findings related to malaria infection.

(1) Comprehensiveness of Experimental Design:

- a) **Diverse Stimuli:** While N67 gDNA and RNA were utilized, these were not the sole components analyzed in our study. Importantly, we included intact, biologically relevant N67 iRBCs as a crucial stimulus. These iRBCs contain a wide range of parasite-specific molecular patterns and represent a more accurate model of natural infection, allowing for a more nuanced understanding of the immune response.
- b) **In Vivo Validation:** We conducted experiments using a mouse model infected with the N67 strain of malaria, providing a direct and physiologically relevant validation of our findings.

(2) Rationale for Control Stimuli: We recognize that N67 gDNA and RNA themselves might primarily elicit a general innate immune response to nucleic acids. Therefore, to distinguish between generic nucleic acid responses and the specific regulation observed during malaria infection, and to validate the universality of the core mechanism, we carefully designed a broad panel of control stimuli (**New Figure 21**): Pathogen infections including VSV (RNA virus), HSV (DNA virus), *Toxoplasma gondii* (*T. gondii*, other parasite control), and pattern molecule stimuli, such as LPS, poly(I:C), and poly(dA:dT), which are known to activate various innate immune pathways.

- a) **Conservation of the Core Pathway:** Our findings indicate that TBK1 depalmitoylation is not exclusive to *Plasmodium* infections. We demonstrated similar effects following stimulation with *T. gondii* and exposure to poly(I:C) and poly(dA:dT). This suggests that TBK1 depalmitoylation serves as a common regulatory mechanism in the immune response to a variety of

nucleic acid-sensing pathogens or danger signals.

- b) **Regulate the Specificity of Enzymes:** Our concurrent analysis of palmitoylating (ZDHHCs) and depalmitoylating enzymes highlighted a significant finding: the expression of specific enzymes (ZDHHC9/APT2) was markedly distinct based on the applied stimulus. This factor underscores the stimulus-specificity of the molecular pathways regulating these enzymes, emphasizing that while TBK1 depalmitoylation is a shared downstream effect, the mechanisms controlling ZDHHC9/APT2 enzyme pair-identified as the core axis in our *Plasmodium* model-exhibit high pathogen specificity.

[editorial note: figure redacted due to its containing unpublished data]

Overall, while our experiments employing N67 qDNA and RNA were aimed at elucidating the role of nucleic acid components in regulatory processes, the principal conclusions pertaining to malaria-specific responses were primarily derived from our *in vitro* experiments involving N67 iRBCs and confirmation through the *in vivo* infection model. Our comparisons across various pathogens and experimental conditions indicate that the modulation of the ZDHHC9/APT2 axis is uniquely associated with malaria infection, reinforcing the specificity of our findings. Thank you for highlighting this important aspect; we hope this detailed rationale adequately addresses the need for appropriate controls in our experimental design.

5. The major concern about the discovery is the lack of transparency of the RNA-seq experiments. How many replicates are used? The heatmap plot shows that a single replicate is only used for each group, and it is strongly recommended to have triplicates to infer the results with more confidence. Maybe a PCA plot can better represent the distribution and number of samples. It is strongly recommended that the raw data be deposited in public nucleotide archives like SRA.

Response: We sincerely thank the reviewer for the valuable comments. The suggestions you provided regarding experimental reproducibility, data presentation, and transparency are of utmost importance. We fully agree with these suggestions and have made the following key improvements based on them:

(1) **Increase biological replicates:** We fully recognize the significance of using an adequate number of biological replicates in RNA-seq analysis to ensure the reliability of the analysis. As suggested, we have re-conducted the experiment and set each group of samples to three independent biological replicates. The current analysis is based on three biological replicates per group (n=3). This setting conforms to the standard operating procedures in this field and significantly enhances the statistical power, reliability, and reproducibility of the experimental results.

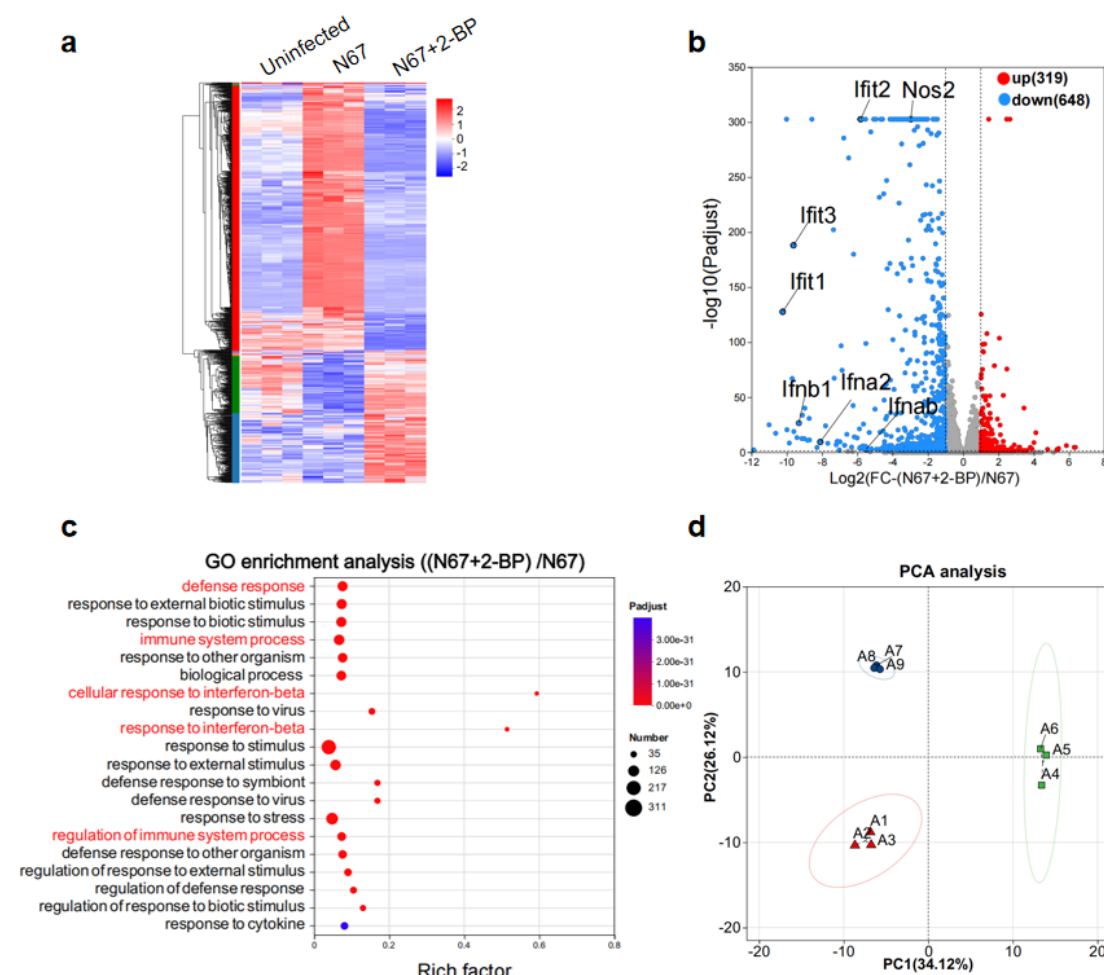
(2) **Update Fig. 1d-f after repeating the experiment:** After repeating the experiment, it was found that 2-BP treatment led to the downregulation of hundreds of genes, including key interferon-stimulated genes (ISGs) involved in the innate immune response against malaria infection (**New Figure 22a,b**). Gene Ontology (GO) analysis further indicates that after 2-BP treatment, genes involved in various immune signaling pathways show a significant enrichment trend, especially those related to the “defense response” and “response to interferon- β ” (**New Figure 22c**).

(3) **Supplementary principal component analysis (PCA):** PCA was performed on the new dataset incorporating three biological replicates per group (**New Figure 22d**). This visualization intuitively confirms the sample size (n=3 per group) and its

distribution, thereby reinforcing the credibility of the experimental findings.

(4) **Make the raw data publicly available:** We have now submitted the raw RNA-seq sequencing data (FASTQ files) containing all three biological replicates to the NCBI Sequence Read Archive (SRA) public database. The data accession number is PRJNA1288125. Reviewers and readers can access this database at any time to obtain the raw data.

The above supplementary experiments and analyses have significantly enhanced the rigor of this study and the persuasiveness of the conclusions. We hope that increasing to three biological replicates, providing PCA visualization results, and making the raw data publicly available have comprehensively and effectively addressed the core concerns you raised. Thank you again for helping us improve this study.



New Figure 22, a-c presented as **Fig. 1d-f** in the revised manuscript. **d** was not shown in the revised manuscript.

(a,b) Heatmap (a) and volcano plot (b) display the differentially expressed genes in peritoneal macrophages (PEMs) pre-treated with DMSO or 2-BP (100 mM for 24 h),

with or without stimulation following freeze-thawed iRBCs. Each group has three biological replicates.

(c) Gene ontology (GO) enrichment analysis of the differentially expressed genes shown in panel b.

(d) Principal Component Analysis (PCA) analysis. Each point represents an individual sample. Colors indicate experimental groups: Uninfected (red, n=3), N67 (green, n=3), N67+2-BP (blue, n=3). The first two principal components (PC1 and PC2) are shown, explaining 34.12% and 26.12% of the total variance, respectively.

6. Another major concern is the approach behind the identification of ZDHHC9. It is mentioned in the figure 2J that at the RNA level, all the family members are dysregulated. Some genes are dysregulated to almost zero. Is it normalized in comparison with uninfected control? If the genes are this much dysregulated upon infection, why are they not present in the RNA-seq results? Maybe a heat map of these family members in RNA-seq can explain this better. Protein level dysregulation can be showed for better clarity.

Response: We sincerely extend our deepest gratitude to the reviewer for providing detailed and professional comments. Your attention to the identification method of ZDHHC9 and the significance of the dysregulation of its family members at the RNA level is highly insightful. In response to your questions, we provide the following replies:

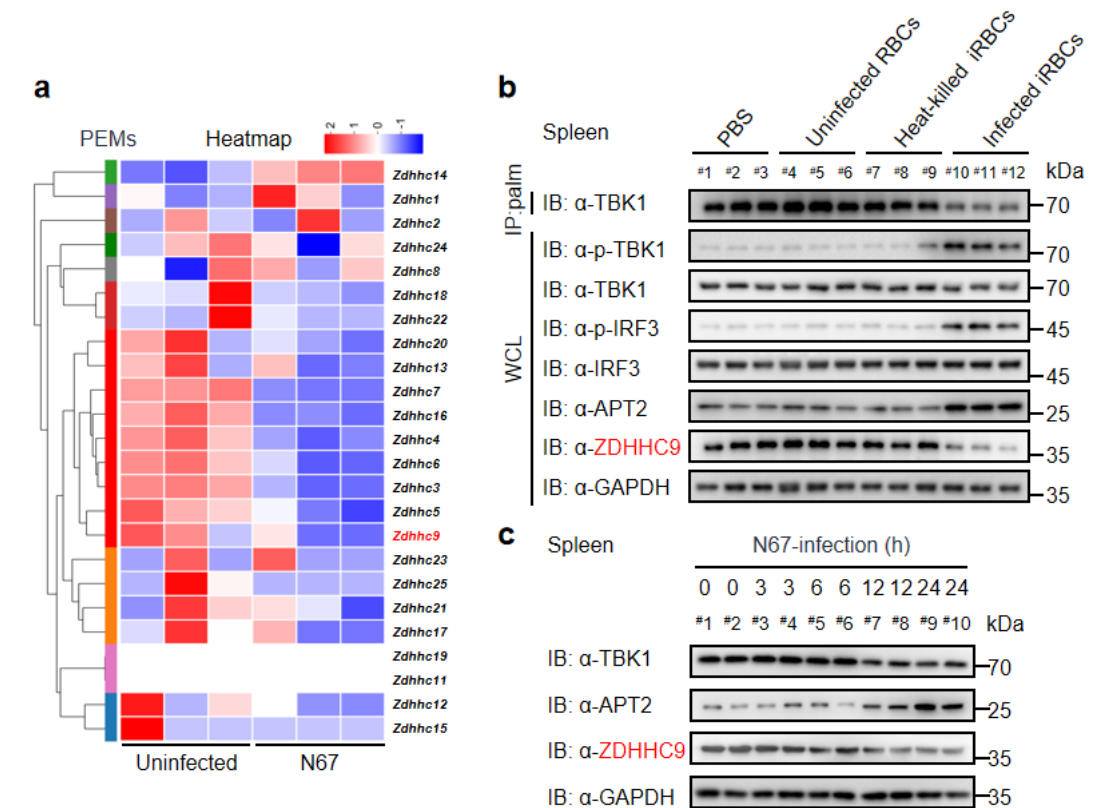
(1) RNA level dysregulation: All the RNA level data presented in figure 2J are based on the comparative analysis between the infected group and the uninfected group. Therefore, the “downregulation” or “upregulation” presented in the figure represents the relative change compared to the uninfected state.

(2) The presentation of ZDHHC9 in the RNA-seq heatmap: Following your valuable suggestion, to more clearly show the overall expression pattern of the ZDHHC family, we have provided a heatmap of the expression of ZDHHC family members based on RNA-seq data (**New Figure 23a**, presented as **Fig. 2g**). This heatmap intuitively shows that under infection conditions, multiple ZDHHC family members indeed exhibit significant transcriptional level dysregulation. Among them, ZDHHC9 clearly shows a downward trend in this heatmap, which is highly consistent with our initial screening results and the expectations of functional studies.

(3) Protein level verification: To further confirm the research findings at the RNA level and evaluate the possible functional consequences, we have supplemented crucial detection results of the ZDHHC9 protein level. As shown in **New Figure 23b,c**, following infection with *Plasmodium yoelii* N67, we observed that the expression level of the ZDHHC9 protein was also significantly downregulated, which is consistent with the downward trend we observed at the RNA level. This result not only strongly

validates the reliability of the RNA-seq and preliminary screening data but also strongly suggests that the function of ZDHHC9 may be inhibited during the infection process.

We sincerely thank you again for your valuable comments and suggestions. These comments and suggestions have greatly enhanced the rigor of our research and the clarity of data presentation. We have clearly added the relevant results in the revised manuscript (Lines 148–150, Page 6).



New Figure 23, a presented as **Fig. 2g** and **c** presented as **Supplementary Fig.6c** in the revised manuscript. **b** was not shown in the revised manuscript.

(a) Heatmap analysis of expression changes in the ZDHHC gene family in PEMs, with or without stimulation following freeze-thawed iRBCs.

(b) C57BL/6J mice (n=6 per group) treated with PBS, uninfected RBCs, heat-killed iRBCs or infected RBCs via intraperitoneal injection for 24 h. Cell lysates derived from spleen were collected for immunoblot analysis.

(c) C57BL/6J mice were infected with N67 at various time points. Protein levels within the spleens were collected for IP (with anti-TBK1) and immunoblot analysis.

7. The mere reduction of the ZDHHC proteins may not correlate with the palmitoylation level of TBK1. The depalmitoylation by increasing the level of APT2 can also be the reason for the reduction of palmitoylation during malarial infection. A coimmunoprecipitation study with the whole family of ZDHHC family members with

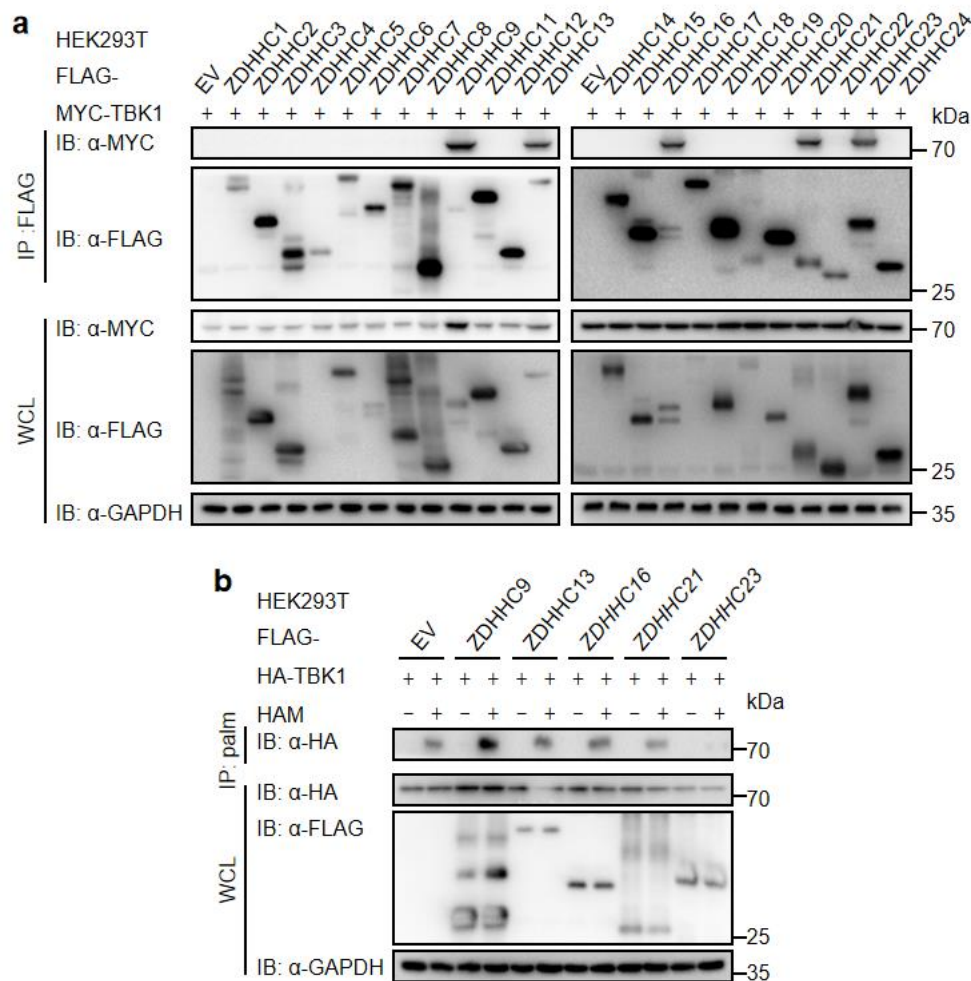
TBK1 could have shed light on this scenario.

Response: We sincerely thank the reviewers for the valuable comments. To effectively respond to the reviewer's suggestions and clearly clarify this issue, we conducted the following key experiments:

(1) **Exogenous Co-IP screening of ZDHHC members and TBK1:** We systematically performed exogenous Co-IP experiments on all ZDHHC family members and TBK1. The experimental results showed that five members, ZDHHC9, ZDHHC13, ZDHHC16, ZDHHC21, and ZDHHC23, could interact with TBK1 exogenously (**New Figure 24a**).

(2) **Functional verification:** To evaluate the functional impact of these interacting members on the palmitoylation of TBK1, we further detected the effect of overexpressing five ZDHHC molecules on the palmitoylation level of TBK1. The experimental results indicated that among these five ZDHHC members interacting with TBK1, only overexpressing ZDHHC9 could significantly increase the palmitoylation level of TBK1. Through further functional experiments, it was confirmed that ZDHHC9 is the key enzyme mediating the upregulation of TBK1 palmitoylation. This research achievement provides important experimental evidence for a deeper understanding of the regulatory mechanism of TBK1 palmitoylation during malaria infection (**New Figure 24b**).

Indeed, we fully agree with the reviewer's view that the de-palmitoylation process mediated by APT2 may also be an important link in regulating TBK1 palmitoylation. Based on this, we propose that ZDHHC9 is the primary enzyme responsible for maintaining TBK1 palmitoylation under resting conditions. However, following malaria infection, the downregulation of ZDHHC9 coupled with the upregulation of APT2 collaboratively further reduces the palmitoylation level of TBK1. Therefore, both APT2 and ZDHHC9 play crucial roles in the dynamic regulatory process of TBK1 palmitoylation. We have clearly added the relevant results in the revised manuscript (Lines 153-159, Page 6).



New Figure 24, presented as **Supplementary Fig. 2d,e** in the revised manuscript.

(a) HEK293T cells were transfected with plasmids encoding FLAG-ZDHHC family members and MYC-TBK1. Cell lysates were collected for IP (with anti-FLAG) and immunoblot analysis.

(b) HEK293T cells were transfected with HA-TBK1 and either FLAG-tagged empty vector (EV), ZDHHC9, ZDHHC13, ZDHHC16, ZDHHC21, or ZDHHC23 for 24 h. TBK1 palmitoylation levels were detected by ABE assay and immunoblot analysis.

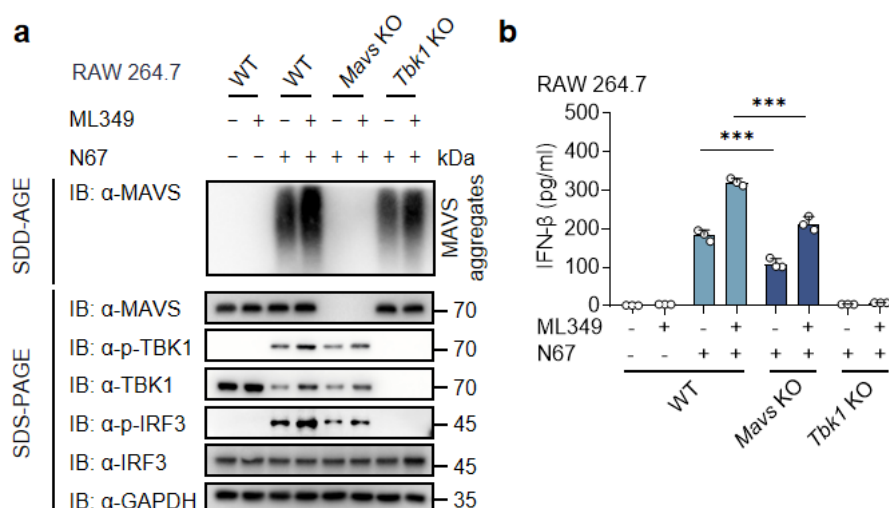
8. It is mentioned that the treatment with APT2 inhibitor ML349 enhances the anti-malarial immune response by elevating the palmitoylation level of TBK1 and increasing IFN-I. However, the inhibition of APT2 by ML349 can affect all of its interactors, not only TBK1. For instance, the upstream adapter MAVS is also affected by ML349, as per various studies.

Response: We appreciate the reviewer's valuable insights. While it is true that ML349, as an APT2 inhibitor, can impact multiple interacting partners, including MAVS, our gene knockout studies for MAVS provide clear evidence that ML349 enhances TBK1 stability independently of MAVS. Here are the key findings supporting this conclusion:

(1) In *Mavs*-KO RAW 264.7 cells, TBK1 protein levels showed a significant increase following ML349 treatment (**New Figure 25a**). This indicates that APT2 inhibition stabilizes TBK1 through a mechanism that does not involve MAVS.

(2) Persistence of downstream signals: In *Mavs*-KO RAW 264.7 cells, while the activation of downstream signals such as p-TBK1 and p-IRF3 was attenuated, ML349 still enhanced the phosphorylation levels of TBK1 and its downstream effectors as well as increased IFN- β production. In contrast, in *Tbk1*-KO RAW 264.7 cells, ML349 failed to enhance IFN-I activation, resulting in undetectable levels of both basal and stimulated IFN-I activation (**New Figure 25a,b**). This demonstrates that ML349 retains its biological activity in stabilizing TBK1 even when MAVS is absent.

In summary, these findings suggest that although APT2 regulates several substrates, including MAVS, its specific role in TBK1 depalmitoylation represents a distinct mechanism that contributes to the inhibition of IFN-I. We have included these results in the revised manuscript (Lines 235-240, Page 8).



New Figure 25, presented as **Fig. 4j** and **Supplementary Fig. 4j** in the revised manuscript.

(a) WT, *Mavs*-KO, or *Tbk1*-KO RAW 264.7 cells were treated with or without ML349 (10 μ M) for 12 h and subsequently stimulated with freeze-thawed iRBCs for 6 h. Cell lysates were collected for immunoblot analysis.

(b) WT, *Mavs*-KO, or *Tbk1*-KO RAW 264.7 cells were treated with or without ML349 (10 μ M) for 12 h and subsequently stimulated with freeze-thawed iRBCs for 6 h. IFN- β release in the supernatants was determined by ELISA.

Minor Concerns:

9. In several figures (fig. 1P, extended fig 1i, 1j), the infection with N67 iRBCs is inducing more response than the N67 RNA or N67 gDNA alone. How is it possible and the authors should shed light on this matter and explain the infection model better.

ISRE luc is lower than IFN luc in few experiments (Supplementary Fig. 2d). What could be the potential reason?

Response: We sincerely appreciate the reviewer's constructive comments.

(1) Compared to isolated nucleic acid components (RNA or gDNA), N67 iRBCs elicit a stronger immune response primarily because they present the complete pathogen structure, including membrane proteins, glycolipids, and nucleic acid-protein complexes. This comprehensive presentation enables a more robust activation of the host immune system. This superiority stems from several key mechanisms:

- a) **Synergistic PRR Activation:** N67 iRBCs engage multiple pattern recognition receptors (PRRs) concurrently. For instance, TLR9 recognizes their genomic DNA (gDNA), TLR7/8 detects their RNA components, and receptors like CLECs bind their surface glycosylated antigens. This multi-pathway synergy is significantly more potent than the primarily single-nucleic-acid-sensing pathways activated by free nucleic acids.
- b) **Mimicry of Natural Infection:** N67 iRBCs infection closely resembles the natural route of pathogen invasion. It involves critical physiological steps such as cellular adhesion, phagocytosis, and antigen processing/presentation. This process not only facilitates the efficient delivery of nucleic acids but also creates a complex microenvironment that simulates an authentic infection.
- c) **Amplification by Danger Signals:** During infection or phagocytosis, damaged host cells or N67 iRBCs may release damage-associated molecular patterns (DAMPs), further amplifying inflammatory signaling. In contrast, artificially transfected free nucleic acids cannot replicate this intricate, multi-step physiological infection process or the diverse danger signals accompanying it. Consequently, they induce a relatively weaker immune response.

(2) We acknowledge that under the specific experimental conditions presented in **Supplementary Figure 2d**, ISRE luciferase activity appears relatively low compared to IFN luciferase activity. We contend that this observation is biologically plausible, primarily attributable to the inherent dynamics and hierarchical organization of the interferon signaling pathway. The likely explanation is: Specifically, the IFN-luciferase reporter gene can directly respond to the major pathogen sensing pathways (such as the activation of IRF3). In contrast, the activation of the ISRE-luciferase reporter gene requires a secondary signaling cascade: the secreted IFN-I must first bind to the IFNAR receptor, thereby activating the JAK/STAT signaling pathway, promoting the formation of the ISGF3 complex, and ultimately driving the activity of the ISRE promoter. Therefore, in these specific experiments related to **Supplementary Fig. 2d**, at a specific time point and/or stimulation intensity, the primary IFN-I response may have been strongly activated, while the secondary ISRE response may still be in the development stage. In some cases, this dependence on IFN-I secretion and IFNAR

signal transduction may result in the ISRE-luc level being lower than the IFN-luc level. Furthermore, we reviewed relevant literature and found instances where ISRE-luciferase activity was reported to be lower than the IFN-luciferase activity^{30, 31}, supporting our interpretation.

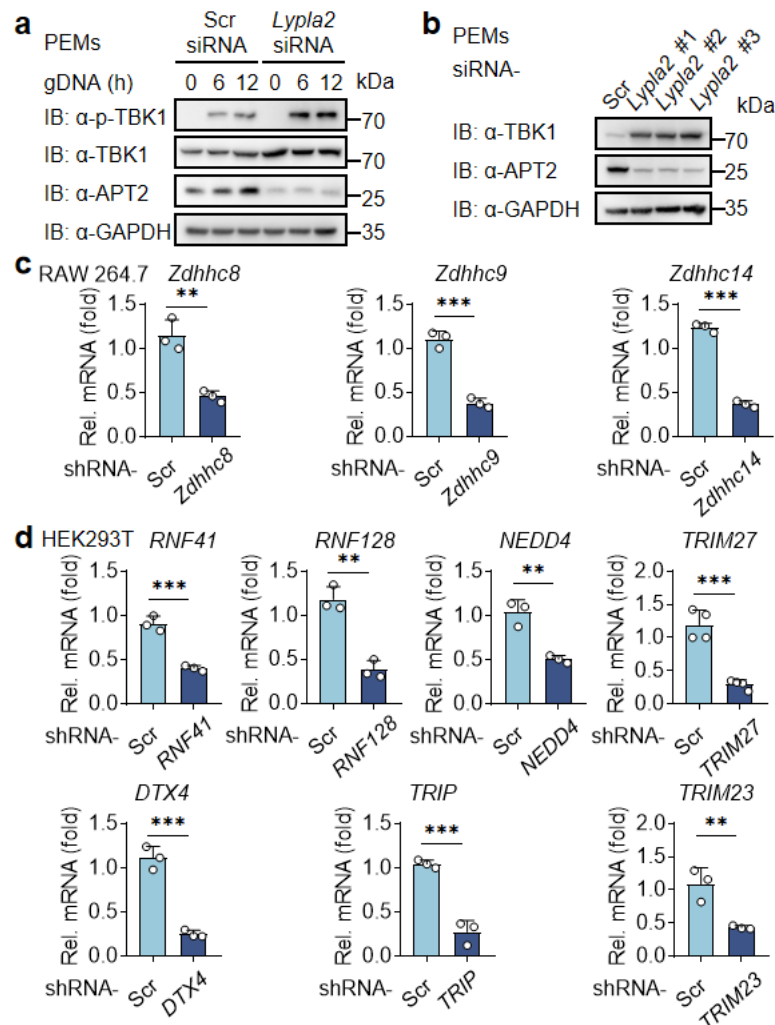
10. The authors should provide data on the knockdown efficiency of siRNAs and shRNAs, as this is crucial for validating the reliability of their gene silencing experiments. Currently, the manuscript does not include any evidence—neither in the main figures nor in the supplementary figures—demonstrating the extent of knockdown at the mRNA or protein level.

Response: We sincerely thank the reviewer for this important and constructive comment. We fully recognize the crucial role of validating the knockdown efficiency of siRNA and shRNA in ensuring the reliability of gene silencing experiments and the accuracy of result interpretation. We conducted the verification alongside the experiments but regret not including these data in the initial manuscript. In the revised version, we have organized and included the relevant data as follows:

(1) **Validation of siRNA Knockdown Efficiency:** The knockdown efficiency of the *Lyp1a2*-siRNA (targeting APT2) used in this study was rigorously validated through multiple independent western blot analyses. We present two representative sets of experimental results in the **New Figure 26a, b**. These data consistently demonstrated that this siRNA effectively and significantly reduced the expression level of APT2 in the employed cell lines. This validation provides a solid molecular foundation for the subsequent functional investigations utilizing this siRNA.

(2) **shRNA knockdown efficiency:** We apologize for the oversight in not clearly presenting the key data for shRNA-mediated stable gene silencing in the initial manuscript. In response to the reviewer's request, we have organized and analyzed these data, which are now included in the revised manuscript (**New Figure 26c,d**). The supplementary data confirm that the shRNA used can effectively and stably knockdown the expression of the target genes, thereby ensuring the reliability of the experimental results obtained from these stable cell lines.

We hope that these supplementary data significantly enhance the rigor and integrity of the manuscript. Once again, we thank the reviewers for the meticulous review and valuable suggestions.



New Figure 26, presented as **Fig. 4k**, and **Supplementary Fig.5b, 2c, 6g** in the revised manuscript.

(a) PEMs were transfected with Scr-siRNA or *Lyp1a2*-siRNA for 36 h, then stimulated with gDNA at indicated time points. Cell lysates were collected for immunoblot analysis.

(b) PEMs were transfected with Scr-siRNA, *Lyp1a2* siRNA #1, #2 or #3 for 36 h. Cell lysates were collected for immunoblot analysis.

(c) RAW 264.7 cells were transfected with Scr shRNA or *Zdhhc* shRNAs for 24 h, and the silencing effect was assessed using RT-qPCR analysis.

(d) HEK293T cells were transfected with Scr shRNA or other E3 ligase-specific shRNAs for 24 h. The silencing effect was assessed using RT-qPCR analysis.

11. Please clarify whether the enzymatically inactive mutants ZDHHC9 (C169S) and APT2 (S122A, C2S) have been previously characterized in published studies. If these mutants have been established in earlier research, proper citations should be provided to support their validity.

Response: We sincerely appreciate the reviewer's valuable suggestion. The enzyme inactivated mutants ZDHHC9 (C169S)³² and APT2 (S122A, C2S)³³ have indeed been characterized in previously published studies. We have now included the relevant citations in the revised manuscript to support the validity of these mutants (References 32 and 33). Thank you for bringing this to our attention.

12. The authors should ensure that the cell lines used in each experiment are clearly indicated in the figures, as was done in the initial figures of the manuscript. Consistently labeling the cell lines throughout the figures would improve clarity and help readers accurately interpret the results.

Response: We appreciate the reviewer's insightful comment regarding the labeling of cell lines in the figures. We apologize for the inconsistency in labeling the cell types across the figures. In the revised manuscript, we have ensured that all relevant figures are uniformly labeled with the corresponding cell lines, maintaining consistency with those presented in the initial figures. Thank you for your valuable feedback, which has helped improve the clarity of our manuscript.

13. The authors should correct the inconsistency between the figure legend, the main manuscript description, and the actual figure. In the legend for Fig. 4e and 4f, as well as in the manuscript text, both IFN β -luc and ISRE-luc are mentioned. However, in the figure itself, only IFN β -luc is shown. The authors should clarify whether ISRE-luc data were included and, if not, revise the text accordingly to accurately reflect the data presented in the figure.

Response: We sincerely appreciate the reviewer for identifying this significant inconsistency. After a thorough re-examination of our data and experimental records, we confirm that no ISRE-luc reporter assays were conducted for the experiments presented in **Fig. 4e** and **4f**. We have revised the figure legend and corresponding text in the manuscript to accurately reflect that only the IFN- β -luc data are included. Thank you for your valuable feedback, which has helped us improve the accuracy of the manuscript.

14. The authors can include an explanation of how parasitemia was calculated, as this is not mentioned in the Methods section. Providing details on the methodology—such as whether parasitemia was assessed by Giemsa staining, flow cytometry, or qPCR—is essential for reproducibility and proper interpretation of the results.

Response: We appreciate the reviewer's constructive comment regarding the methodology for calculating parasitemia. We apologize for not providing sufficient detail in the Methods section. Clearly defining the measurement method is indeed

essential for ensuring both reproducibility and accurate interpretation of our results. In this study, parasitemia was assessed through microscopic examination of Giemsa-stained blood smears³⁴. We have now included a detailed description of this experimental method in the “Methods” section of the revised manuscript (Lines 692-704, Page 22). Thank you for your valuable feedback.

Once again, we sincerely thank the reviewers for their rigorous and meticulous review and constructive comments.

Responses to Reviewer #4:

Reviewer #4: 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.

Response: We thank the reviewer very much for the insightful and thorough comments. The co-reviewed concerns have been addressed above.

References:

1. Liu, Z. *et al.* Ubiquitylation of autophagy receptor Optineurin by HACE1 activates selective autophagy for tumor suppression. *Cancer Cell* **26**, 106-120 (2014).
2. Jiao, Y. *et al.* Enteric coronavirus nsp2 is a virulence determinant that recruits NBR1 for autophagic targeting of TBK1 to diminish the innate immune response. *Autophagy* **20**, 1762-1779 (2024).
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Response to the reviewers concerning revised manuscript

Responses to Reviewer #1:

Reviewer #1:

My comment has been resolved.

Response: We sincerely thank the reviewer for their thoughtful feedback and for helping us improve our manuscript.

Responses to Reviewer #2:

Reviewer #2:

The authors successfully addressed my concerns. I believe this is a strong manuscript.

Response: We sincerely thank the reviewer for the positive evaluation of our revised manuscript.

Responses to Reviewer #3:

Reviewer #3:

In the revised manuscript titled "Palmitoylation of TBK1 enhances the type I interferon signaling and strengthens anti-malarial immunity," the authors have adequately addressed all the concerns raised during review. The manuscript is in a strong and polished form, with the content, structure, and presentation meeting the standards required for publication.

Response: We are grateful for the reviewer's thoughtful comments and their recognition of the revisions we have made.

Responses to Reviewer #4:

Reviewer #4:

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.

Response: We appreciate the reviewer's constructive feedback, which has been instrumental in strengthening our manuscript.