nature portfolio

Peer Review File

The nutrient-sensing Rag-GTPase complex in B cells controls humoral immunity via TFEB/TFE3-dependent mitochondrial fitness

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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)
In this manuscript, Zhu et al. described how Rag-GTPases, independent of mTORC1, modulate amino acid sensing and mitochondrial metabolism in B cells to support their development and function. They demonstrated that B cell-specific deletion of RagA/RagB disrupts GC formation, antibody production, and plasmablast generation, implicating abnormal TFEB/TFE3 activity and mitophagy dysregulation. Additionally, the manuscript differentiated the roles of TFEB/TFE3 in TD and TI humoral responses and outlines the distinct metabolic requirements for these processes. The authors have demonstrated these results using different mouse models and chimera mouse models in the process of arriving at their conclusions, as well as studying the roles of mTORC1 and RagA/B in different developmental stages of B cells, including spleen and GC B cells. The observation of non-RagA/B-dependent amino acid sensing is intriguing. However, the novelty and physiological significance are lacking, and the underlying mechanism by which Rag-GTPases regulate TFEB/TFE3 activity remains unclear. Please see detailed comments below.

Major points:
1. The observations of impaired B cell development resulting from either TFEB/TFE3 overactivation or mTORC1 deficiency, as demonstrated in other studies (e.g., PMID: 22709692, 31676673, 31729420, 31533002, etc.), diminish the novelty of the findings presented in this manuscript. While the authors demonstrate that Rag-GTPase deficiency leads to TFEB/TFE3 overactivation and subsequent mitochondrial and immunological deficiencies, the molecular details of how RagA/B regulate TFEB/TFE3 activity remain somewhat unclear. It appears that RagA/B deficiency mirrors the phenotype of FLCN-FNIP deficiency in B cells (PMID: 22709692, 31676673). Further elucidation of this regulatory network may enhance the mechanistic novelty of the study.

2. The authors compared and identified distinct phenotypes between RagA/B- and Raptor-deficient B cells. However, their conclusion that the phenotypes in RagA/B-deficient B cells do not depend on mTORC1 signaling lacks logical coherence. To support this conclusion, the authors should explore the effects of inhibiting or depleting mTORC1 signaling specifically in the context of RagA/B deficiency.

3. The evidence linking the B cell development defect to TFEB/TFE3 overactivation in the context of RagA/B deficiency is convincing in this study, but lacking strong evidence to demonstrate the role of mitophagy in mediating this defect. Does inhibition of lysosomal activity or mitophagy rescue the phenotype of RagA/B-deficient B cells?

4. The observation that amino acids modulate mTORC1 signaling in a RagA/B-independent manner is intriguing. Given its significance as a major novelty in this manuscript, the authors could delve deeper into this point, discussing potential regulatory mechanisms by which amino acids regulate mTORC1 signaling bypassing Rag-GTPase and offering speculative insights in the discussion part.

Minor points:
1. Line numbers should be added to the manuscript for easier review.

2. The manuscript requires careful proofreading. For example, the title of the first part of Results, "Amino acids modulates

mTORC1 independent of Rag-GTPases" is grammatically incorrect. Consider revising to "Amino acids modulate mTORC1 independently of Rag-GTPases" for clarity and grammatical correctness.

3. The manuscript might benefit from a clearer explanation regarding specific terms and abbreviations upon their first occurrence. For example, explaining "AID" the first time it is mentioned could aid readers unfamiliar with the terminology.

4. “Later stage B cells, including B220+CD43– pre-B cells/immature B cells and B220hiCD43– circulating mature B cells, were mostly reduced in the absence of RagA/RagB (Fig. 2A).” B220+ includes both B220hi and B220lo; was there a mistake, and should it be B220lo?

5. The interpretation order of Figure 2 could be improved. It initially discusses Figure 2A/B, then jumps to C/D, and back to Figure 2A/B, making it difficult to follow.

6. In Figure S2B, there was no significant difference between the WT and experimental groups in Fraction B, so it should not be stated that there were significant differences.

Reviewer #2

(Remarks to the Author)
In this manuscript, the authors investigated the roles of Rag-GTPase (RagA/RagB) complexes in murine B cell development and humoral immunity using the CreLoxP system. In response to B cell activation and nutrient availability, B cells undergo rapid metabolic reprogramming to accommodate B cell growth and division, germinal center formation, and eventually antibody production. Rag-GTPases sense amino acid availability to modulate and link nutrients to mTORC1 activation and cellular growth. mTORC1, in addition to driving protein and lipid synthesis, also phosphorylates the MiT/TFE transcription factors TFEB and TFE3, resulting in nuclear exclusion and prevention of lysosome biogenesis. However, how Rag-GTPases coordinate amino acid sensing with mTORC1 activation and TFEB/TFE3 regulation during B cell activation and humoral immunity had not been previously defined. The authors show that Rag-GTPases are important for normal B cell development and activation, GC formation, affinity maturation, and TI and TD antibody production. Mechanistically, RagGTPases A and B constrain TFEB/TFE3 activity to prevent mitophagy dysregulation and maintain mitochondrial fitness, which surprisingly seem to occur independent of mTORC1 regulation. Importantly, deletion of TFEB/TFE3 rescued B cell development, GC formation, and TI immunity in RAG-GTPase deficient B cells.
Overall, the manuscript and experiments performed were very thorough and the authors went to great lengths to support their conclusions. However, one major issue is that they failed to consider, is that there is a documented feedback response of TFE3/TFEB hyperactivation in stimulating mTORC1 activity, as had been shown in other systems (see Napolitano et al “Non-canonical mTORC1 signaling at the lysosome” Trends in Cell Biology 2022 for review). In particular, the GAP activity of Folliculin (FLCN) is required for activation of RagC/D-GDP, which in turn is required for phosphorylation of TFEB/TFE3 by mTORC1. In the absence of RagA/B, the RagGTPase complex may be unable allow mTORC1 to phosphorylate TFEB/TFE3, leading to constitutive nuclear location. Once activated, TFEB/TFE3 promote non-canonical mTORC1 activation through a negative feedback loop which relies on increased transcription of RagC/D GTPases (see Alesi et al “TFEB drives mTORC1 hyperactivation and kidney disease in Tuberous Sclerosis Complex” Nature Communications 2023). This may explain the paradoxical activation of mTORC1 in the absence of RagA/B in B cells.

Comments

(1) In the absence of RagA/B in B cells, RagD is transcriptionally upregulated (Fig. 5D). Does deletion of TFEB/TFE3 restore RagC/D transcription to normal levels? If RagC/D is deleted in RagA/B deficient B cells, does this prevent mTORC1 activation?

(2) In the introduction, there is no mention of the regulation of MiTF/TFE family members by FLCN/FNIP pathway, which is an important aspect of recruitment to the lysosome, and deletion of FLCN/Fnip1 is also important for B cell development (Park et al., Disruption of Fnip1 reveals a metabolic checkpoint controlling B lymphocyte development” Immunity 2012; Baba et al.,The Folliculin-FNIP1 pathway deleted in human Birt-Hogg-Dube syndrome is required for B cell development” Blood 2012). Because FLCN is upregulated in CreERRagAfl/fl RagBfl/fl B cells, this could be another feedback response in attempt to recruit mTORC1 to phosphorylate TFEB/TFE3. Is FLCN recruited to the lysosome in the absence of RagA/B?

(3) For constitutively active (CA) TFEB, what was the relative activation of mTORC1? Similarly, for the CreERRagAfl/fl RagBfl/fl x TFE3/TFEB dKO, what was the relative activation of mTORC1 (p-S6, p-4EBP1) before and after deletion of MiTF factors? Because expression of RagD and RagC are increased in CreERRagAfl/fl RagBfl/fl mice, one might expect mTORC1 to be reduced following MiTF inactivation. If mTORC1 is reduced, this could imply that RagA/B factors are important for canonical mTORC1 activation, but not non-canonical mTORC1 activation driven by nuclear MiTF.

(4) Throughout the manuscript, there is really no mention of the effects of RagA/B deletion on cell survival, which could affect the interpretation of many of the experiments (i.e. differentiation vs survival).

Minor comments
(1) In Figure 2, why are the B220hiCD43- B cells depleted in RagA/B KO mice but not Raptor KO mice following tamoxifen injection? Does depletion of Rags impair survival of long-lived recirculating FO B cells? This raises the question as to whether other effects shown (such as GC formation) are due in part to reduced survival rather than GC formation per se.

(2) In Figure 1C, RagA/B deletion led to increased p-S6R relative to WT B cells, suggesting that mTORC1 activation may be higher. Similarly, immunoblotting results (Figure 1D) also suggest that mTORC1 activation may be higher following RagA/B deletion. This may be consistent with non-canonical activation of mTORC1?

Reviewer #3

(Remarks to the Author)
In this study entitled “The nutrient-sensing Rag-GTPase complex in B cells controls humoral immunity via TFEB/TFE3-dependent mitochondrial fitness”, the authors used a genetic approach to investigate the roles of Rag-GTPases and mTORC1 in B cell development and function in vivo. The findings revealed that acute deletion of either RagA/RagB or Raptor impairs early B cell development. It was also shown that B cell-intrinsic Rag-GTPases are critical for germinal center (GC) formation in Peyer's patches, proper distribution between dark zones (DZ) and light zones (LZ), and both T-dependent (TD) and T-independent (TI) antigen-induced antibody responses, despite these GTPases having a minimal role in mTORC1 activity.

The mechanism underlying these effects involves Rag-GTPases suppressing the activity of transcription factors TFEB and TFE3, largely independently of canonical mTORC1 activity. This suppression is necessary to maintain mitochondrial fitness by reducing lysosomal activity and preventing abnormal mitophagy in B cells. Consequently, deletion of TFEB/TFE3 helps restore mitochondrial function, GC formation in Peyer's patches, and humoral immunity for TI antigens but does not completely recover the response to TD antigen immunization when Rag-GTPases are absent.

Collectively, the study suggests that Rag-GTPases contribute to B cell function and development by suppressing TFEB/TFE3 activity, ensuring mitochondrial health, and supporting humoral immune responses, with these roles being largely independent of of mTORC1.

Overall, this is an interesting wide-ranging study that addresses, and provides valuable insights into, the complex role of RagA/RagB GTPases at different stages of B cell development and function.

The following points need to be clarified:

- The authors use ROSA26-Cre-ERT2 to acutely delete the studied genes. However, there are no data on the deletion efficiency. It is known that inducible deletion depends on the targeted gene and on the tissue. Thus, it would be important to provide molecular data for the efficiency of the acute gene inactivation of the individual genes.
- What is the rationale behind the protocol for the acute deletion? In the several Figure legends the authors state: “Tamoxifen was administered to animals intraperitoneally daily for 4 consecutive days. Splenic B cells were purified 7 days after the last tamoxifen injection”. In the methods, the authors write that mice were analyzed 8 days after the last injection. This should be corrected.
- What was the genotype of the control (WT) mice? Were they always Cre-ER as stated in the Figure legend 3. If the controls were always the same why do the B cell phenotypes show considerable differences? For instance, the WT FACS plots in Fig 2A and B differ dramatically. Were the control mice also treated with tamoxifen?
- In almost all CD43/B220 FACS plots, the numbers are covering the cell populations in the Figures making it difficult to evaluate the data.
- Minor point: The abbreviation for differentially expressed genes (DEGs) is give as acronym on page 11 and explained on page 14.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed my questions.

Reviewer #2

(Remarks to the Author)
The authors made a good effort to experimentally address the comments from the reviewers. However, there are some concerns regarding interpretation of the data that they should address prior to publication:

(1) Just because the authors see further inhibition of IgG1 expression with Rapamycin treatment (Figure S1F), does not mean that RagA/RagB depletion is acting independently of mTORC1. If mTORC1 inhibition is incomplete following RagA/RagB deletion, the phenotypes may be further altered by Rapamycin. Similarly, because constitutively active TFEB may drive increased mTORC1 activation (Figure S6E), this suggests that the minimal reduction of mTORC1following RagA/B deletion could be due to increased nuclear TFEB and non-canonical activation of mTORC1. Finally, the rapamycin

treatment studies (Figure 1G) are difficult to interpret. While it is true that rapamycin inhibits mTORC1, rapamycin has also been shown to paradoxically decrease phosphorylation of TFEB, resulting in nuclear exclusion (see PMID: 38195686). Hence, the changes seen following Rapa treatment of RagA/B deficient B cells are difficult to interpret.

(2) The authors appear to ignore the comments from reviewer 1 and 2 regarding the already documented roles of FLCN/Fnip1 in regulating phosphorylation of TFEB in response to RagGTPases (for example, see PMID: 32612235). In particular, In FLCN deficient cells, RagC (and mTORC1) is unable to bind and phosphorylate TFEB resulting in nuclear location and constitutive activation similar to RagA/B deficient cells. In addition, FLCN is also largely dispensible for phosphorylation of S6K and 4E-BP1 by mTORC1 (also similar to RagA/B deficient cells). Given the important roles of Fnip1/FLCN in B cell development and activation in response to nutrients, it is quite possible that the mechanism of TFEB constitutive nuclear localization in Rag dKO mice is due to improper regulation of the FLCN/Fnip1 pathway. This should be mentioned in the discussion. It is not surprising that the phenotypes of FLCN and Rag dKO mice are not identical, because they have both proteins likely have overlapping and independent functions.

(3) Figure 1- font way too small to read
(4) The question regarding if RagC/D is deleted in RagA/B deficient B cells, does this prevent mTORC1 activation was not really answered. Although published models suggest that full activation of Rag GTPase requires both RagA/RagB-GTP and RagC/RagD-GDP, their data suggest that RagA/B KO B cells have normal p-4EBP1 and reduced p-S6K and p-S6R. In addition, transcriptional activation of RagD has been shown to drive mTORC1 activation and cancer growth (PMID: 28619945, PMID: 31225432, PMID: 35654731)

(5) Reference 1 in the rebuttal is incorrect. I believe it should be PMID: 31676673

(6) In Rebuttal Figure 1A, what do the symbols refer to in the graph (left)? Need legend

(7) Lines 39-41 in the abstract " ….our data establish …. As an mTORC1 independent mechanism to coordinating nutrient sensing and mitochondrial metabolism" is overstated.

(8) Line 81 "cytokine" should be "cytokines".

(9) Lines 97-99: I believe "independent of mTORC1" is overstated. Similar comments regarding line 426

Reviewer #3

(Remarks to the Author)
The authors addressed the previous concerns and questions adequately. With the new experiments and modifications, the clarity and robustness of the findings is greatly enhanced. Based on these extensive revisions, I believe the manuscript is now significantly improved and acceptable for publication.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my concerns/questions

Overall, all three reviewers found your work timely and interesting. They raised concerns, however, on the lack of validation for some of the mechanistic hypotheses. Although we expect all reviewer criticisms to be addressed, we consider it particularly important that you further validate the mTORC1-independent regulation B of cells by RagA/B and you provide deeper mechanistic insight on how the RagA/B function might be compensated. Please examine the potential role of the FLCN/FNIP pathway as R#1 and 2 suggest and explain the compensatory upregulation of mTORC1 activity as per R#2. Please provide the controls and methodical details about the mouse genetics experiments according to the suggestions of R#3.

When resubmitting, you must provide a point-by-point response to the reviewers' comments. Please show all changes in the manuscript text file with track changes or colour highlighting. If you are unable to address specific reviewer requests or find any points invalid, please explain why in the point-by-point response.

We are very grateful for the positive assessment and the guidance for the revision. We have performed extensive experiments that addressed all the concerns raised by the reviewers. Please see our point-by-point response below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, Zhu et al. described how Rag-GTPases, independent of mTORC1, modulate amino acid sensing and mitochondrial metabolism in B cells to support their development and function. They demonstrated that B cell-specific deletion of RagA/RagB disrupts GC formation, antibody production, and plasmablast generation, implicating abnormal TFEB/TFE3 activity and mitophagy dysregulation. Additionally, the manuscript differentiated the roles of TFEB/TFE3 in TD and TI humoral responses and outlines the distinct metabolic requirements for these processes. The authors have demonstrated these results using different mouse models and chimera mouse models in the process of arriving at their conclusions, as well as studying the roles of mTORC1 and RagA/B in different developmental stages of B cells, including spleen and GC B cells. The observation of non-RagA/B-dependent amino acid sensing is intriguing. However, the novelty and physiological significance are lacking, and the underlying mechanism by which Rag-GTPases regulate TFEB/TFE3 activity remains unclear. Please see detailed comments below.

We thank the reviewer for the critical evaluation of our manuscript.

Major points:

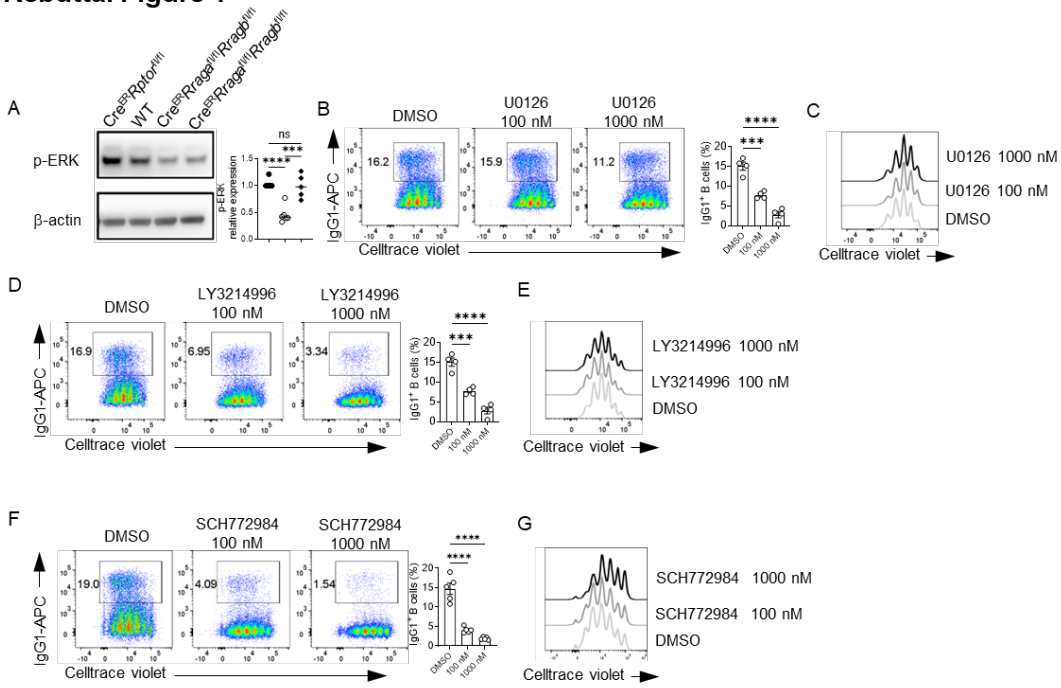
1. The observations of impaired B cell development resulting from either TFEB/TFE3 overactivation or mTORC1 deficiency, as demonstrated in other studies (e.g., PMID: 22709692, 31676673, 31729420, 31533002, etc.), diminish the novelty of the findings presented in this manuscript. While the authors demonstrate that Rag-GTPase deficiency leads to TFEB/TFE3 overactivation and subsequent mitochondrial and immunological deficiencies, the molecular details of how RagA/B regulate TFEB/TFE3 activity remain somewhat unclear. It appears that RagA/B deficiency mirrors the phenotype of FLCN-FNIP deficiency in B cells (PMID: 22709692, 31676673). Further elucidation of this regulatory network may enhance the mechanistic novelty of the study.

We appreciate the reviewer's critique and suggestions. While previous studies have demonstrated that loss of FLCN-FNIP complex or Raptor (mTORC1) severely impairs B cell development, the functions of Rag-GTPase in B cell development remain less understood. Furthermore, although one of them (*1*) reported that increased mTORC1 activation and TFE3 nuclear localization are **associated** with Fnip1 deficiency in B cell precursors, it did not establish causal relationship between TFE3 activation and Fnip1 deletion induced B cell defects. By contrast, our study untangles the divergent contribution of Rag-GTPase and mTORC1 to B cell development and differentiation and more importantly established the **causal relationship** between increased TFEB/TFE3 activity and Rag-GTPase deficiency induced B cell defects (at least part of the defects). Finally, our preliminary data indicate that FLCN and Rag-GTPase may have distinct functions in B cells (see data below), indicating that one may not extrapolate observations from FLCN-FNIP1 mutant mice to Rag-GTPase mutant mice. We hope that the reviewer will concur.

While we have not completely figured out how Rag-GTPase regulates TFEB/TFE3 activity, we found that ERK pathway, which is known to modulate TFEB activity (*2-4*), was downregulated in

Rag GTPase deficient B cells, but not in Raptor deficient B cells (Rebuttal Figure 1A). Moreover, multiple ERK pathway inhibitors dose-dependently inhibited IgG1 expression on B cells without significantly affecting B cell proliferation (Rebuttal Figure 1B-1G), which was reminiscent of Rag GTPase deficient B cells. ERK can phosphorylate TFEB on Ser142 (*2*). We have demonstrated that Ser142 and Ser211 mutations significantly increase TFEB/TFE3 activity and reduce IgG1 class switching (Figure 6B). Thus, ERK pathway could be a potential target that is controlled by Rag-GTPase. We will further investigate the role of the ERK pathway and other possible targets controlled by Rag-GTPase in the future.

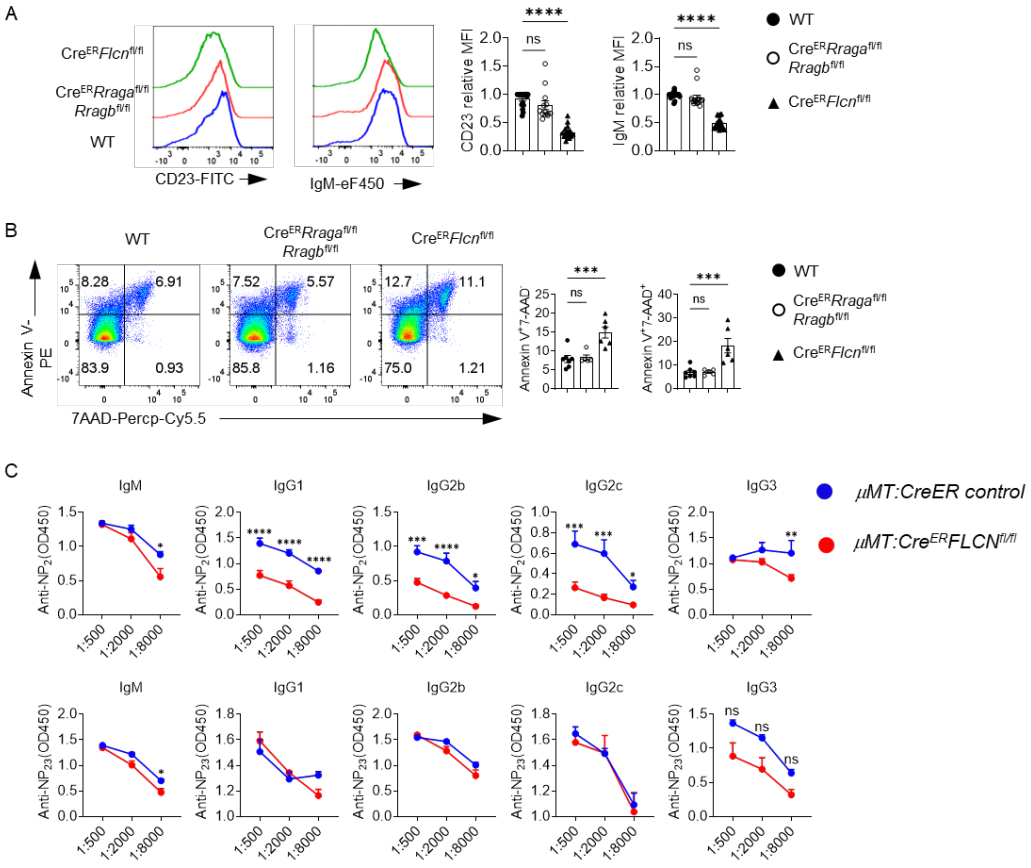
Rebuttal Figure 1



Rebuttal Figure 1. ERK modulates B cell activation. (A) p-ERK level was examined on the B cells activated with LPS/IL-4/BAFF for 72 h by immunoblot. (B-G) WT B cells were labeled with Celltrace Violet (CTV) and stimulated with LPS/IL-4/BAFF in the presence of ERK inhibitors. (B) Representative flow plots of CTV and IgG1 expression on B cells treated with different concentrations of ERK inhibitor U0126. Right, a summary of the percentage of $IgG1^+$ B cells. (C) Representative flow plots of CTV. (D) Representative flow plots of CTV and IgG1 expression on B cells treated with different concentrations of ERK inhibitor LY3214996. Right, a summary of the percentage of $IgG1^+$ B cells. (E) Representative flow plots of CTV. (F) Representative flow plots of CTV and IgG1 expression on B cells treated with different concentrations of ERK inhibitor SCH772984. Right, a summary of the percentage of $IgG1^+$ B cells. (G) Representative flow plots of CTV. Data in graphs represent mean ± SEM. ns, not significant. ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA (A, B, D and F).

Regarding FLCN mediated B cell functions, we have also analyzed FLCN deficient mice. We found that FLCN deficient B cells had distinct phenotypes compared to RagA/RagB deficient B cells. FLCN deficiency, but not Rag GTPase deficiency, highly reduced the expression of CD23 and IgM on B cells (Rebuttal Figure 2A). FLCN deficient B cells, but not RagA/B deficient B cells, showed a significant increase in apoptosis after overnight activation (Rebuttal Figure 2B). Furthermore, the humoral immune response in B cell-specific FLCN deficient mice was quite different from that in RagA/RagB deficient mice. Antigen-specific all affinity antibody titers remained intact in FLCN deficient mice, while the high-affinity antibody titers were reduced (Rebuttal Figure 2C) (whereas RagA/RagB deficient mice have reduced antibody titers for all affinity antibodies and high affinity antibodies, Figure 3D-3E). Overall, our results indicate that FLCN and Rag-GTPase may modulate B cell development and activation through distinct mechanisms. We are actively investigating this topic.

Rebuttal Figure 2



Rebuttal Figure 2. FLCN regulates B cell function differently from Rag GTPase. (A-B) Tamoxifen was administered to animals intraperitoneally daily for 4 consecutive days. Mice were sacrificed and analyzed 7 days after the last injection. (A) CD23 and IgM levels were measured on splenic B cells. Right, Summaries of relative MFI of CD23 and IgM (WT B cell CD23 and IgM expression was set as "1"). (B) B cells were activated overnight with LPS/IL-4/BAFF and Annexin V and 7-AAD were examined. Right, summaries of the percentages of Annexin V^+7-

AAD$^-$ and Annexin V$^-$7-AAD$^+$. (C) Tamoxifen was administered to animals by oral gavage daily for 4 consecutive days. Mice were immunized intraperitoneally (100 μg NP-OVA/alum) 7 days after the last injection. Serum NP-specific all affinity antibody titers (NP_{23}) and high-affinity antibody titers (NP_2) were measured by ELISA. Data in graphs represent mean ± SEM. ns, not significant. **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA (A and B), two-way ANOVA (C).

2. The authors compared and identified distinct phenotypes between RagA/B- and Raptor-deficient B cells. However, their conclusion that the phenotypes in RagA/B-deficient B cells do not depend on mTORC1 signaling lacks logical coherence. To support this conclusion, the authors should explore the effects of inhibiting or depleting mTORC1 signaling specifically in the context of RagA/B deficiency.

Thank you for your insightful comments. We would like to clarify the implication of our data. Because RagA/B KO B cells exhibit largely normal canonical mTORC1 activity (unlike RagA/B KO Tregs (*5*)), and many distinct in vivo and in vitro phenotypes compared to Raptor KO B cells, it is reasonable to propose that Rag-GTPase may regulate B cells independent of mTORC1. However, we are also mindful that the distinctions are not absolute, as indicated in Fig. 2C-2E, Fig. 3A, 3D, 3E, 3H-3I, 3K-3N. Furthermore, at 72h after stimulation, RagA/B KO B cells exhibited modestly reduced p-S6, although p-4EBP1 was still preserved (new Figure S1F). Thus, we do not exclude the possibility that under certain circumstances Rag-GTPase might operate in a partially mTORC1 dependent manner. We have modified our texts to indicate such nuance.

Nonetheless, we have performed additional experiments to test if mTORC1 inhibition, mediated by rapamycin treatment, can exacerbate B cell activation defects in the absence of Rag-GTPase. Indeed, we found that rapamycin could magnify the IgG1 expression defects between RagA/RagB deficient B cells and WT B cells in a dose dependent manner (new Figure 1G), suggesting that both Rag-GTPase and mTORC1 contribute to IgG1 expression at least in a partially non-redundant manner. However, rapamycin did not further amplify the defects of TMRM and MTDR staining in the absence of Rag-GTPase, nor did it affect TMRM and MTDR staining in WT B cells (new Figure S4A-S4B), suggesting that these mitochondrial phenotypes were controlled by Rag-GTPase, but not mTORC1. Altogether, these data are consistent with our hypothesis that Rag-GTPase can modulate certain mitochondrial phenotypes independent of mTORC1.

Figure S1F

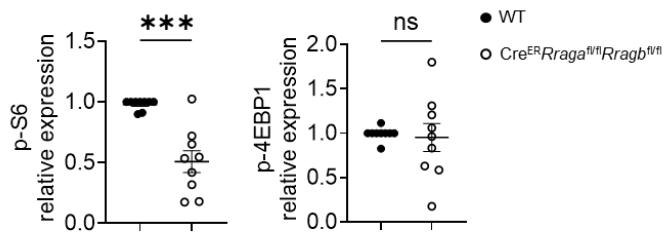


Figure S1F. Summaries of p-S6 and p-4EBP1 expressions in the B cells stimulated with LPS/IL-4/BAFF for 72 h and examined by immunoblot. Data in graphs represent mean ± SEM. ns, not significant. ***p < 0.001, two-tailed student T-test was used.

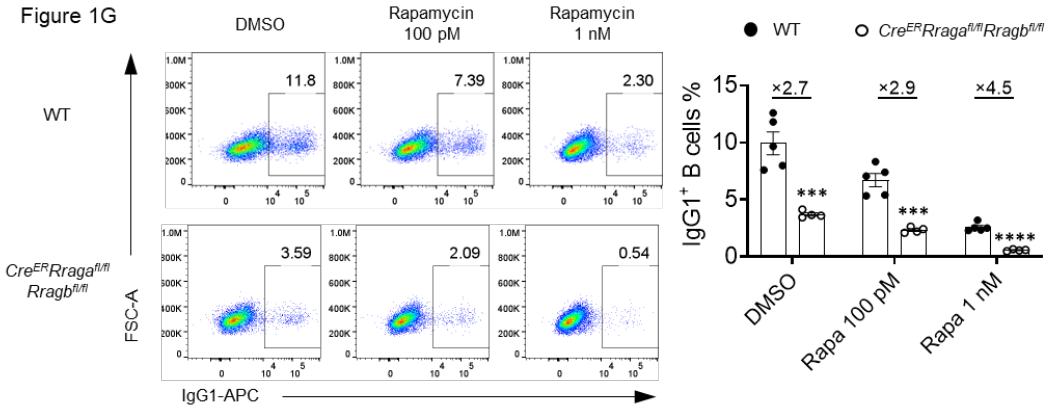


Figure 1G. Rapamycin was added into the culture medium at 24 h after B cells were stimulated with LPS/IL-4/BAFF, and IgG1 expression was examined by flow cytometry after B cells were stimulated for additional 48 h. Right, summary of the percentages of $IgG1^+$ B cells. The numbers indicate the fold differences of average $IgG1^+$ percentages between WT and RagA/RagB deficient B cells. WT mice (n = 5), $Cre^{ER}Rraga^{fl/fl}Rragb^{fl/fl}$ mice (n = 4). Data in graphs represent mean ± SEM. ***p < 0.001, ****p < 0.0001, two-tailed Student's t test was used.

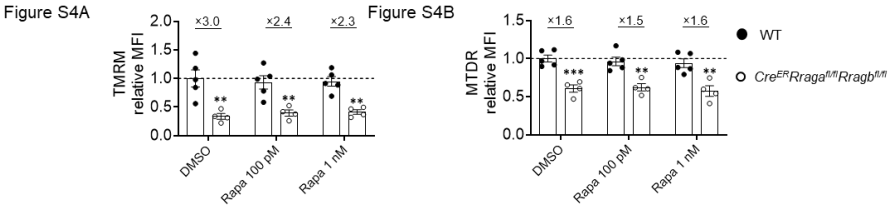


Figure S4A-S4B. Rapamycin was added into the culture medium at 24 h after B cells were stimulated with LPS/IL-4/BAFF. TMRM (Figure S4A) or MTDR (Figure S4B) staining was examined by flow cytometry after B cells were stimulated for additional 48 h. Data in graphs represent mean ± SEM. **p < 0.01, ***p < 0.001, two-tailed Student's t test.

3. The evidence linking the B cell development defect to TFEB/TFE3 overactivation in the context of RagA/B deficiency is convincing in this study, but lacking strong evidence to demonstrate the role of mitophagy in mediating this defect. Does inhibition of lysosomal activity or mitophagy rescue the phenotype of RagA/B-deficient B cells?

Thank you for your thoughtful suggestions. To directly test how mitophagy modulates B cell in general, we treated WT B cells with different mitophagy activators including Urolitin A, MF094 and CMPD-39. We found that the mitophagy activators significantly reduced the IgG1 expression without affecting B cell proliferation (new Figure S5A-S5B). Conversely, IgG1 expression was enhanced when mitophagy was inhibited by different mitophagy inhibitors, including 3-MA, IC-87114 and PRT062607 (new Figure S5C-S5F). Importantly, mitophagy inhibitors could partially rescue the IgG1 expression defects in RagA/B deficient B cells (new Figure 5P). Collectively, these data indicate that abnormal mitophagy overactivation is partially responsible for the defective class switch *in vitro* in the absence of Rag-GTPase, and excessive mitophagy could be detrimental to B cell activation.

Figure S5

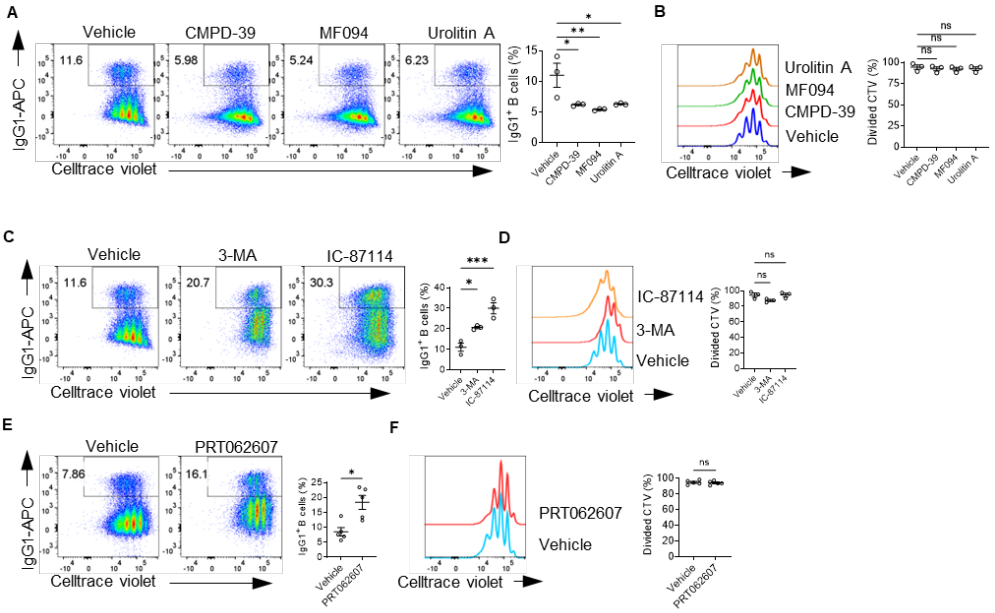


Figure 5P

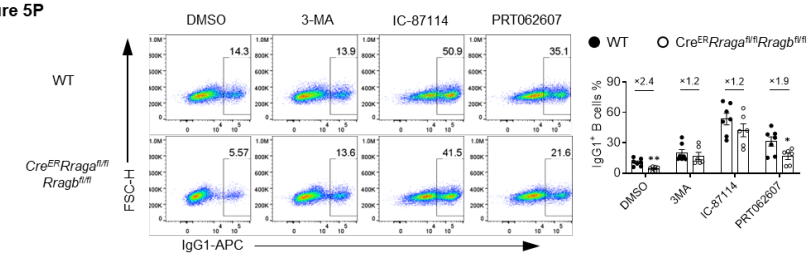


Figure S5. Mitophagy modulates B cell activation. (A and B) B cells were labeled with Celltrace violet (CTV) and stimulated with LPS/IL-4/BAFF in the presence of mitophagy activators CMPA-39 (1 mM), MF094 (1 mM) or Urolitin A (1 mM) for 72 h, IgG1 expression (A) and dilution of CTV (B) were examined by flow cytometry. Right, the percentage of $IgG1^+$ B cells (A) or divided CTV (B). (C-F) B cells were labeled with CTV and stimulated with LPS/IL-4/BAFF and mitophagy inhibitors 3-MA (1 mM), IC-87114 (5 mM) (C and D) or PRT062607 (1

mM) (E and F) for 3 days, IgG1 expression and CTV dilution were measured by flow cytometry. **Figure 5P.** B cells were stimulated with LPS/IL-4/BAFF and indicated mitophagy inhibitors including 3-MA (1 mM), IC-87114 (5 mM) and PRT062607 (1 mM) for 3 days, the percentage of $IgG1^+$ B cells was examined by flow cytometry. Right, summary of the percentages of $IgG1^+$ B cells. WT mice (n = 7), $Cre^{ER}Rraga^{fl/fl}Rragb^{fl/fl}$ mice (n = 6). Data in graphs represent mean ± SEM. ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001, and, one-way ANOVA (Figure S5A-S5D), two-tailed student T-test (Figure S5E-S5F), two-tailed Student's t test (Figure 5P).

4. The observation that amino acids modulate mTORC1 signaling in a RagA/B-independent manner is intriguing. Given its significance as a major novelty in this manuscript, the authors could delve deeper into this point, discussing potential regulatory mechanisms by which amino acids regulate mTORC1 signaling bypassing Rag-GTPase and offering speculative insights in the discussion part.

We thank the reviewer for bringing up this direction. Because amino acids modulate both Rag-GTPase and mTORC1, it is indeed plausible that different amino acids have differential effects on GTPase and mTORC1 activation. Such upstream signaling modulation will be an important question for future investigation. We have included our perspective on this aspect in the discussion (Line 434-438). We are also actively working on this topic in our ongoing research.

Minor points:

1. Line numbers should be added to the manuscript for easier review.

Thank you! We have added the line numbers.

2. The manuscript requires careful proofreading. For example, the title of the first part of Results, "Amino acids modulates mTORC1 independent of Rag-GTPases" is grammatically incorrect. Consider revising to "Amino acids modulate mTORC1 independently of Rag-GTPases" for clarity and grammatical correctness.

We are sorry for these oversights! We have carefully revised and proofread the manuscript.

3. The manuscript might benefit from a clearer explanation regarding specific terms and abbreviations upon their first occurrence. For example, explaining "AID" the first time it is mentioned could aid readers unfamiliar with the terminology.

We have added the abbreviations (Line 136, 267, 270) in the manuscript.

4. "Later stage B cells, including B220+CD43– pre-B cells/immature B cells and B220hiCD43– circulating mature B cells, were mostly reduced in the absence of RagA/RagB (Fig. 2A)." B220+ includes both B220hi and B220lo; was there a mistake, and should it be B220lo?

It should be $B220^{lo}$. We have corrected it in the manuscript (Line 146). Thank you!

5. The interpretation order of Figure 2 could be improved. It initially discusses Figure 2A/B, then jumps to C/D, and back to Figure 2A/B, making it difficult to follow.

Thank you for your suggestions. We have revised our manuscript to make it clearer (Lines 146-154).

6. In Figure S2B, there was no significant difference between the WT and experimental groups in Fraction B, so it should not be stated that there were significant differences.

Thank you for pointing out this mistake. We have changed our description as “modestly reduced frequencies of fraction B and C/C’ B cells (Fig. S2B)” (line 172).

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors investigated the roles of Rag-GTPase (RagA/RagB) complexes in murine B cell development and humoral immunity using the CreLoxP system. In response to B cell activation and nutrient availability, B cells undergo rapid metabolic reprogramming to accommodate B cell growth and division, germinal center formation, and eventually antibody production. Rag-GTPases sense amino acid availability to modulate and link nutrients to mTORC1 activation and cellular growth. mTORC1, in addition to driving protein and lipid synthesis, also phosphorylates the MiT/TFE transcription factors TFEB and TFE3, resulting in nuclear exclusion and prevention of lysosome biogenesis. However, how Rag-GTPases coordinate amino acid sensing with mTORC1 activation and TFEB/TFE3 regulation during B cell activation and humoral immunity had not been previously defined. The authors show that Rag-GTPases are important for normal B cell development and activation, GC formation, affinity maturation, and TI and TD antibody production. Mechanistically, RagGTPases A and B constrain TFEB/TFE3 activity to prevent mitophagy dysregulation and maintain mitochondrial fitness, which surprisingly seem to occur independent of mTORC1 regulation. Importantly, deletion of TFEB/TFE3 rescued B cell development, GC formation, and TI immunity in RAG-GTPase deficient B cells.

Overall, the manuscript and experiments performed were very thorough and the authors went to great lengths to support their conclusions. However, one major issue is that they failed to consider, is that there is a documented feedback response of TFE3/TFEB hyperactivation in stimulating mTORC1 activity, as had been shown in other systems (see Napolitano et al “Non-canonical mTORC1 signaling at the lysosome” Trends in Cell Biology 2022 for review). In particular, the GAP activity of Folliculin (FLCN) is required for activation of RagC/D-GDP, which in turn is required for phosphorylation of TFEB/TFE3 by mTORC1. In the absence of RagA/B, the RagGTPase complex may be unable allow mTORC1 to phosphorylate TFEB/TFE3, leading to constitutive nuclear location. Once activated, TFEB/TFE3 promote non-canonical mTORC1 activation through a negative feedback loop which relies on increased transcription of RagC/D GTPases (see Alesi et al “TFEB drives mTORC1 hyperactivation and kidney disease in Tuberous Sclerosis Complex” Nature Communications 2023). This may explain the paradoxical activation of mTORC1 in the absence of RagA/B in B cells.

We appreciate the reviewer's positive evaluation on the rigor of our manuscript.

Comments

(1) In the absence of RagA/B in B cells, RagD is transcriptionally upregulated (Fig. 5D). Does deletion of TFEB/TFE3 restore RagC/D transcription to normal levels? If RagC/D is deleted in RagA/B deficient B cells, does this prevent mTORC1 activation?

We have examined the mRNA level of TFEB downstream targets including *Rragd*, *Rragc*, *Lamp1* and *Flcn* in Cre^{ER}*Rraga*$^{fl/fl}$*Ragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ B cells. Their expression was all significantly restored when TFEB was deleted (Figure S6L).

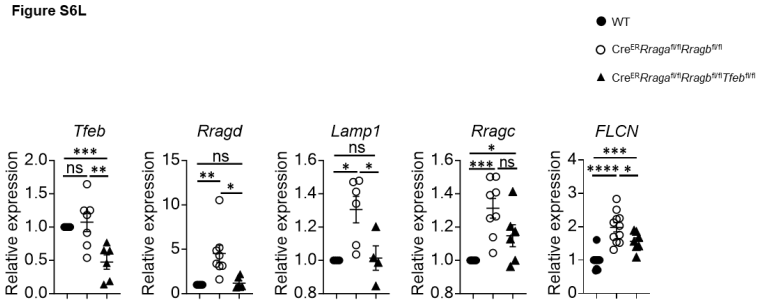


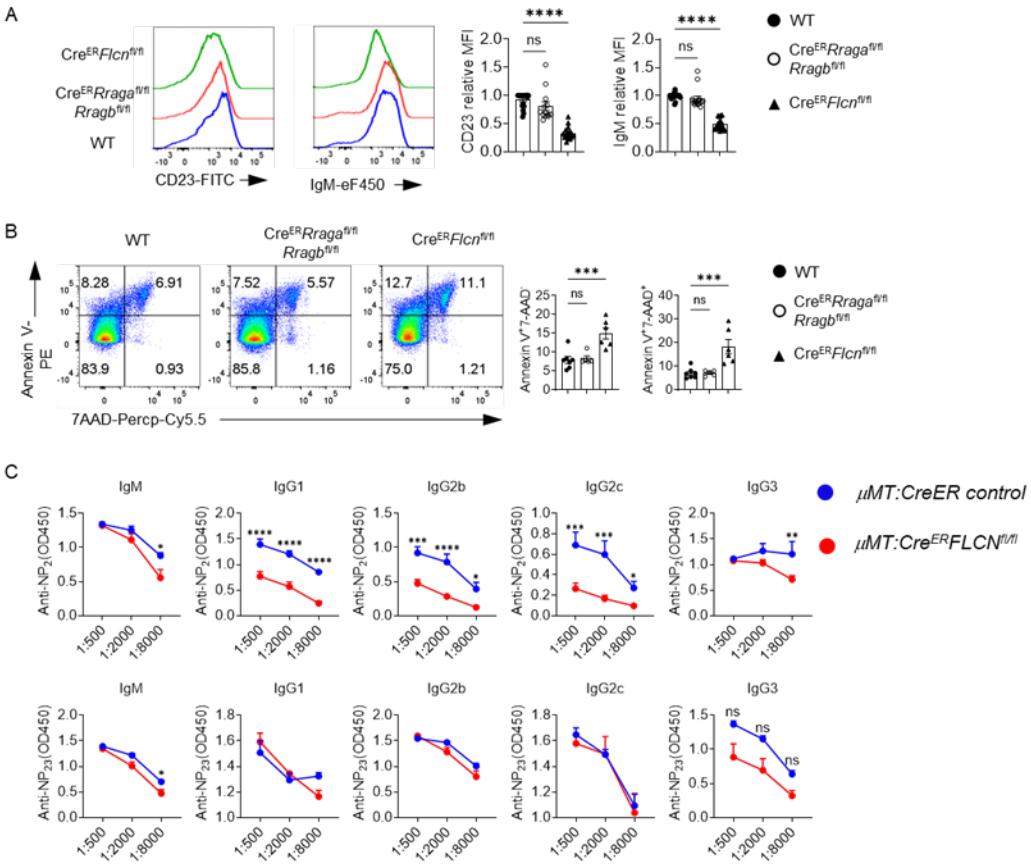
Figure S6L. TFEB downstream target genes were measured on the activated B cells from WT (n = 4-6), Cre^{ER}*Rraga*$^{fl/fl}$*Ragb*$^{fl/fl}$ (n = 6-8), and Cre^{ER}*Rraga*$^{fl/fl}$*Ragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ (n = 4-6) mice. Data in graphs represent mean ± SEM. ns, not significant. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, one-way ANOVA was used.

According to the established model, Rag GTPases function as heterodimers that consist of RagA or RagB in complex with RagC or RagD. The full activation of Rag GTPase complex requires both RagA/RagB-GTP and RagC/RagD-GDP (*6, 7*). Thus, we do not expect that increased *Rragc* and *Rragd* expression can lead to full activation of Rag-GTPase and subsequent activation of mTORC1 in the absence of RagA and RagB. Indeed, our data demonstrated that RagA/B KO B cells have largely normal p-4EBP1 level (Figure 1C, 1D, 3F, Figure S1F), but context dependent impact on p-S6 level (Figure 1C, 1D, 3F, Figure S1E).

(2) In the introduction, there is no mention of the regulation of MiTF/TFE family members by FLCN/FNIP pathway, which is an important aspect of recruitment to the lysosome, and deletion of FLCN/Fnip1 is also important for B cell development (Park et al., Disruption of Fnip1 reveals a metabolic checkpoint controlling B lymphocyte development" Immunity 2012; Baba et al.,The Folliculin-FNIP1 pathway deleted in human Birt-Hogg-Dube syndrome is required for B cell development" Blood 2012). Because FLCN is upregulated in CreERRagAfl/fl RagBfl/fl B cells, this could be another feedback response in attempt to recruit mTORC1 to phosphorylate TFEB/TFE3. Is FLCN recruited to the lysosome in the absence of RagA/B?

We thank the reviewer for bringing up this important issue. It is certainly plausible that increased FLCN is a response to the loss of Rag-GTPase. However, as indicated in above response (to the comment #1), RagA/RagB KO B cells do not have obviously overactivation of mTORC1, but they do have greatly increased TFEB/TFE3 nuclear localization (Figure 5I, Figure S4H). These data indicate that increased FLCN expression is unable to recruit mTORC1, or phosphorylate TFEB/TFE3, or rescue any of the B cell functional defects in the absence of Rag-GTPase. Furthermore, as shown in Rebuttal Figure 2, FLCN deficient B cells exhibit distinct phenotypes compared to RagA/B deficient B cells, including reduced expression of IgM, CD23, increased apoptosis and normal all affinity anti-hapten antibody production in an NP-OVA immunization model. Thus, it is likely that FLCN and Rag-GTPase have nonredundant roles in B cell biology, which is the focus of our ongoing research.

Rebuttal Figure 2

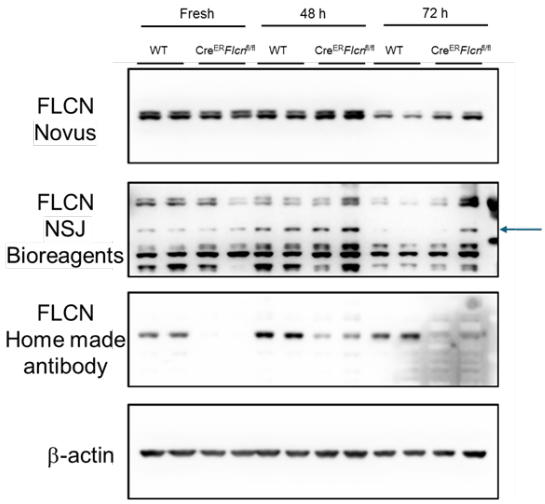


Rebuttal Figure 2. FLCN deficiency and RagA/RagB deficiency have distinct impacts on B cells. (A-B) Tamoxifen was administered to animals intraperitoneally daily for 4 consecutive days. Mice were sacrificed and analyzed 7 days after the last injection. (A) CD23 and IgM levels were measured on splenic B cells. Right, Summaries of relative MFI of CD23 and IgM (WT B cell CD23 and IgM expression was set as "1"). (B) B cells were activated overnight with LPS/IL-4/BAFF and Annexin V and 7-AAD were examined. Right, summaries of the percentages of Annexin $V^+7\text{-}AAD^-$ and Annexin $V^-7\text{-}AAD^+$. (C) Tamoxifen was administered to animals by oral

gavage daily for 4 consecutive days. Mice were immunized intraperitoneally (100 μg NP-OVA/alum) 7 days after the last injection. Serum NP-specific all affinity antibody titers (NP_{23}) and high-affinity antibody titers (NP_2) were measured by ELISA. Data in graphs represent mean ± SEM. ns, not significant. **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA (A and B), two-way ANOVA (C).

Nevertheless, we have tried to find a proper FLCN antibody for immunofluorescence of endogenous FLCN in RagA/B KO B cells. However, so far, none of the commercially available FLCN antibodies can reliably detect endogenous FLCN (Rebuttal Figure 3). Please note that the control FLCN antibody was a home-made mouse antibody that was gift from a collaborator at NIH and not suitable for immunofluorescence. Moreover, we have already run out of it.

Rebuttal Figure 3



Rebuttal Figure 3. B cells from WT or FLCN-deficient mice were used to test the specificity of FLCN antibodies that are claimed suitable for immunofluorescent experiments from different vendors.

(3) For constitutively active (CA) TFEB, what was the relative activation of mTORC1? Similarly, for the CreERRagAfl/fl RagBfl/fl x TFE3/TFEB dKO, what was the relative activation of mTORC1 (p-S6, p-4EBP1) before and after deletion of MiTF factors? Because expression of RagD and RagC are increased in CreERRagAfl/fl RagBfl/fl mice, one might expect mTORC1 to be reduced following MiTF inactivation. If mTORC1 is reduced, this could imply that RagA/B factors are important for canonical mTORC1 activation, but not non-canonical mTORC1 activation driven by nuclear MiTF.

Thank you for your question. We have observed slightly increased levels of p-S6 and p-4EBP1 in the ca TFEB transduced B cells (Figure S6E).

Figure S6E

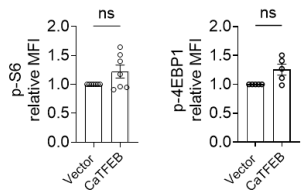


Figure S6E. p-S6 and p-4EBP1 levels were measured on GFP^{+} B cells by flow cytometry and the relative MFI was presented. Data in graphs represent mean ± SEM. ns, not significant. two-tailed student T-test was used.

We also examined p-S6 and p-4EBP1 in both Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ and Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ *Tfe3*$^{-/-}$ B cells. We didn't observe significant changes of p-S6 and p-4EBP1 levels (Figure S6M and Revision Figure 4). Therefore, these data indicate that TFEB and TFE3 may not be a strong activator of mTORC1 in B cells, and they are not critically required for mTORC1 activation in B cells.

Figure S6M

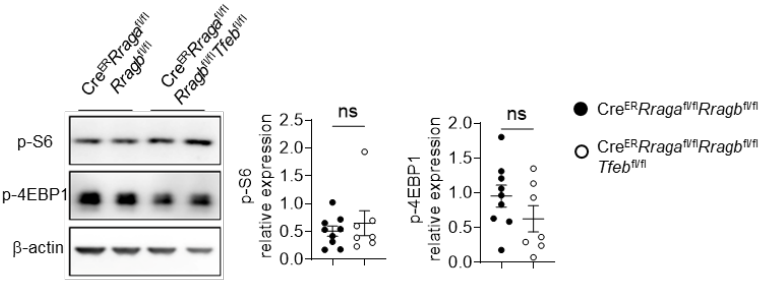
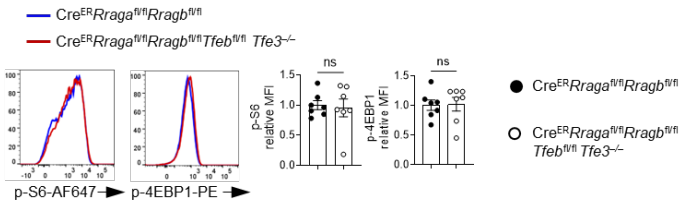


Figure S6M. B cells were activated with LPS/IL-4/BAFF for 72 h and harvested for immunoblot to measure p-S6 and p-4EBP1 level. Right, summary of the p-S6 and p-4EBP1 levels. Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$ (n = 9), and Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ (n = 7). Data in graphs represent mean ± SEM. ns, not significant, two-tailed student T-test was used.

Rebuttal Figure 4

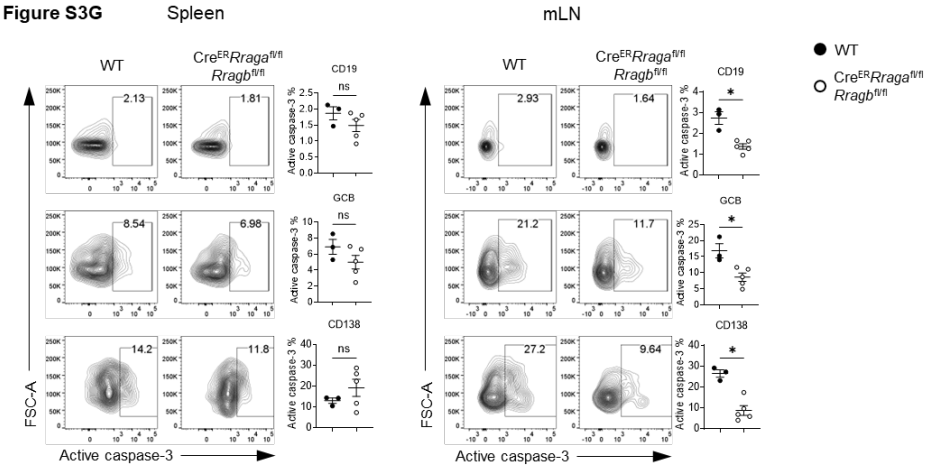


Rebuttal Figure 4. Representative flow plots of p-S6 or p-4EBP1expression on activated B cells from Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$ or Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$*Tfe3*$^{-/-}$ mice. Right, Summaries of

the relative MFI of p-S6 or p-4EBP1 (MFI in the RagA/RagB deficient B cells was set as "1"). Data in graphs represent mean ± SEM. ns, not significant, two-tailed Student's t test was used.

(4) Throughout the manuscript, there is really no mention of the effects of RagA/B deletion on cell survival, which could affect the interpretation of many of the experiments (i.e. differentiation vs survival).

We have performed active caspase-3 staining on RagA/B deficient B cells both in vivo and in vitro. We found that RagA/RagB deficient B cells had no significant change of apoptosis in spleen, and a modest reduction of apoptosis in mLN (new Figure S3G). During in vitro activation, there was no significant change of Annexin-V/7AAD staining in either RagA/B deficient or RagA/B/TFEB/TFE3 KO B cells (new Figure S1G). Thus, Rag-GTPase deficiency does not lead to increased apoptosis in B cells.



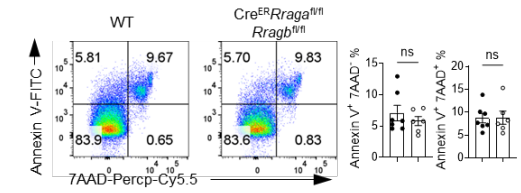


Figure S3G. Active caspase-3 was measured on CD19$^+$, GC and CD138$^+$ B cells of spleens and mesenteric lymph nodes (mLN) from immunized chimera mice. **Figure S1G**. B cells were stimulated with LPS/IL-4/BAFF for 24 h, Annexin V and 7-ADD were examined by flow cytometry. Right, the percentages of Annexin V$^+$7-AAD$^-$ and Annexin V$^+$7-AAD$^+$ B cells. Data in graphs represent mean ± SEM. ns, not significant. *p < 0.05, two-tailed student T-test.

Minor comments

(1) In Figure 2, why are the B220hiCD43- B cells depleted in RagA/B KO mice but not Raptor KO mice following tamoxifen injection? Does depletion of Rags impair survival of long-lived

recirculating FO B cells? This raises the question as to whether other effects shown (such as GC formation) are due in part to reduced survival rather than GC formation per se.

Although we do not have a definitive answer for why the B220hiCD43- B cells are depleted in RagA/B KO mice but not in Raptor KO mice following tamoxifen injection, we observed an association between CXCR4 expression and B220hiCD43- B cell phenotypes in RagA/B and Raptor KO mice. CXCR4 expression was significantly elevated in Raptor KO B220hiCD43- B cells, but significantly downregulated in RagA/B KO B220hiCD43- B cells (new Figure S1H). CXCR4 is a key chemokine receptor that promotes B cell retention in bone marrow (*8, 9*). Our data suggest that Rag-GTPase and mTORC1 could have opposing function on CXCR4 expression on bone marrow circulating B cells, which warrants further investigation.

Figure S1H

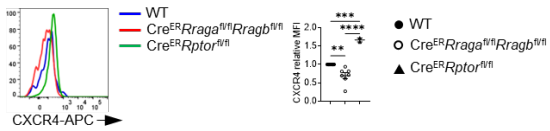


Figure S1H. Expression of CXCR4 on BM B220^{hi}CD43^{-} B cells. Right, the relative MFI of CXCR4 level on BM B220^{hi}CD43^{-} B cells, CXCR4 MFI on WT B220^{hi}CD43^{-} B cells was set as "1". WT mice (n = 7), *Cre^{ER}Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$ (n = 7), and *Cre^{ER}Rptor*$^{fl/fl}$ (n = 2). Data in graphs represent mean ± SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA was used.

For the GC survival, we did not observe significant change of apoptosis of the GC B cells in the spleen of RagA/B KO immunized mice (new Figure S3G).

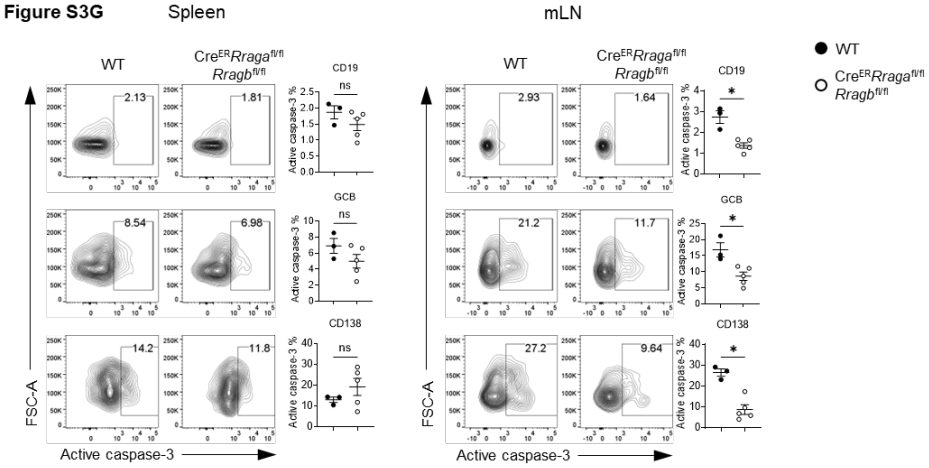


Figure S3G. Active caspase-3 was measured on CD19^{+}, GC and CD138^{+} B cells of spleens and mesenteric lymph nodes (mLN) from immunized chimera mice. Data in graphs represent mean ± SEM. ns, not significant. *p < 0.05, two-tailed student T-test.

(2) In Figure 1C, RagA/B deletion led to increased p-S6R relative to WT B cells, suggesting that mTORC1 activation may be higher. Similarly, immunoblotting results (Figure 1D) also suggest

that mTORC1 activation may be higher following RagA/B deletion. This may be consistent with non-canonical activation of mTORC1?

Although RagA/RagB KO B cells exhibited a slight increase of p-S6 at 24 hours (Figure 1C and 1D), they had a modest reduction of p-S6, but normal p-4EBP1, at 72 hours after activation (new Figure S1F). RagA/RagB KO B cells also did not exhibit significant change in p-S6 and p-4EBP1 ex vivo after NP-OVA immunization (Figure 3F).

Figure S1F

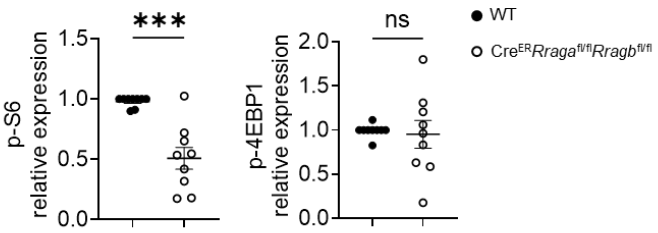


Figure S1F. Summaries of p-S6 and p-4EBP1 expressions in the B cells stimulated with LPS/IL-4/BAFF for 72 h and examined by immunoblot. Data in graphs represent mean ± SEM. ns, not significant. ***p < 0.001, two-tailed student T-test.

Furthermore, we observed no significant changes in p-S6 and p-4EBP1 when TFEB or TFEB/TFE3 were deleted in RagA/B KO B cells (new Figure S6M and Rebuttal Figure 4). Thus, our data do not support any substantial non-canonical mTORC1 activation in RagA/B

Figure S6M

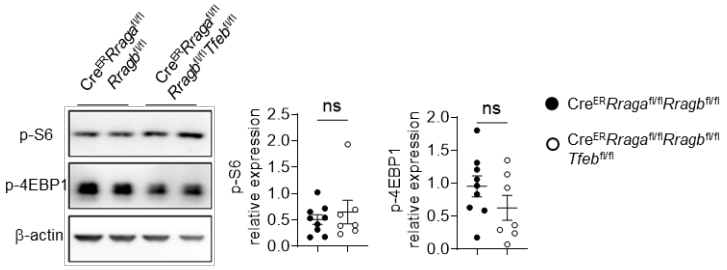
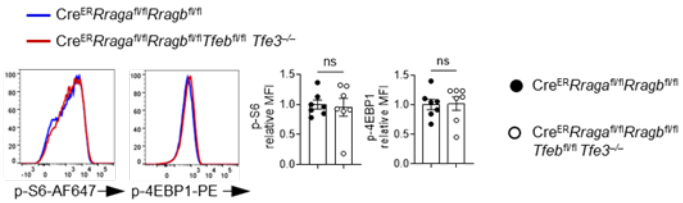


Figure S6M. B cells were activated with LPS/IL-4/BAFF for 72 h and harvested for immunoblot to measure p-S6 and p-4EBP1 level. Right, summary of the p-S6 and p-4EBP1 levels. Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$ (n = 9), and Cre^{ER}*Rraga*$^{fl/fl}$*Rragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$ (n = 7). Data in graphs represent mean ± SEM. ns, not significant, two-tailed student T-test was used.

Rebuttal Figure 4



Rebuttal Figure 4. Representative flow plots of p-S6 or p-4EBP1expression on activated B cells from Cre^{ER}*Rraga*$^{fl/fl}$*Ragb*$^{fl/fl}$ or Cre^{ER}*Rraga*$^{fl/fl}$*Ragb*$^{fl/fl}$*Tfeb*$^{fl/fl}$*Tfe3*$^{-/-}$ mice. Right, Summaries of the relative MFI of p-S6 or p-4EBP1 (MFI in the RagA/RagB deficient B cells was set as "1"). Data in graphs represent mean ± SEM. ns, not significant, two-tailed Student's t test was used.

Reviewer #3 (Remarks to the Author):

In this study entitled "The nutrient-sensing Rag-GTPase complex in B cells controls humoral immunity via TFEB/TFE3-dependent mitochondrial fitness", the authors used a genetic approach to investigate the roles of Rag-GTPases and mTORC1 in B cell development and function in vivo. The findings revealed that acute deletion of either RagA/RagB or Raptor impairs early B cell development. It was also shown that B cell-intrinsic Rag-GTPases are critical for germinal center (GC) formation in Peyer's patches, proper distribution between dark zones (DZ) and light zones (LZ), and both T-dependent (TD) and T-independent (TI) antigen-induced antibody responses, despite these GTPases having a minimal role in mTORC1 activity.

The mechanism underlying these effects involves Rag-GTPases suppressing the activity of transcription factors TFEB and TFE3, largely independently of canonical mTORC1 activity. This suppression is necessary to maintain mitochondrial fitness by reducing lysosomal activity and preventing abnormal mitophagy in B cells. Consequently, deletion of TFEB/TFE3 helps restore mitochondrial function, GC formation in Peyer's patches, and humoral immunity for TI antigens but does not completely recover the response to TD antigen immunization when Rag-GTPases are absent.

Collectively, the study suggests that Rag-GTPases contribute to B cell function and development by suppressing TFEB/TFE3 activity, ensuring mitochondrial health, and supporting humoral immune responses, with these roles being largely independent of of mTORC1.

Overall, this is an interesting wide-ranging study that addresses, and provides valuable insights into, the complex role of RagA/RagB GTPases at different stages of B cell development and function.

The following points need to be clarified:

- The authors use ROSA26-Cre-ERT2 to acutely delete the studied genes. However, there are no data on the deletion efficiency. It is known that inducible deletion depends on the targeted gene and on the tissue. Thus, it would be important to provide molecular data for the efficiency of the acute gene inactivation of the individual genes.

Thank you for pointing out this oversight. We have confirmed the deletion of RagA, TFEB, TFE3 and Raptor on the protein level (new Figure S1A-S1B)

Figure S1

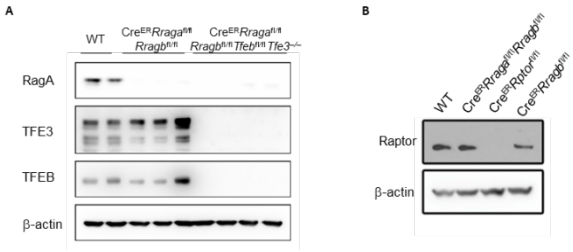


Figure S1. Expression of RagA, TFEB, TFE3 (A) and Raptor (B) was examined by immunoblot. B cells from the indicated genotypes were stimulated with LPS/IL-4/BAFF for 72 h.

- What is the rationale behind the protocol for the acute deletion? In the several Figure legends the authors state: “Tamoxifen was administered to animals intraperitoneally daily for 4 consecutive days. Splenic B cells were purified 7 days after the last tamoxifen injection”. In the methods, the authors write that mice were analyzed 8 days after the last injection. This should be corrected.

We appreciate your careful reading of the manuscript. Our previous publication has demonstrated the efficacy of this regimen in the same mice (*10*). Other publications have used similar strategy for the acute induction of gene deletion (*11, 12*). We mainly analyzed the mice 7 days after the last injection. We have updated this information in the manuscript.

- What was the genotype of the control (WT) mice? Were they always Cre-ER as stated in the Figure legend 3. If the controls were always the same why do the B cell phenotypes show considerable differences? For instance, the WT FACS plots in Fig 2A and B differ dramatically. Were the control mice also treated with tamoxifen?

We always use Cre-ER+ mice as the control mice. All control mice were also treated with tamoxifen in the exact same fashion as the experimental mice. This is mainly to control for any potential Cre toxicity, which has been documented (*13*). Several factors may have contributed to the staining pattern differences. The B cell development data from Raptor KO mice was generated 2-3 years before that from RagA/RagB KO mice. They were acquired on different flow cytometry instruments (BD FACS LSRII vs Attune Nxt) by different investigators. Second, the mice were housed in different institutions when the B cell developmental data was acquired. All these could lead to subtle differences in the flow patterns. Despite these differences, the

frequencies of the B cell precursor subsets had a very comparable range between WT control mice for Raptor KO mice and WT control mice for RagA/RagB KO mice (comparing Figure 2A to Figure 2B), indicating that the flow pattern differences are most likely technical, but not biological. They do not compromise the veracity of the data.

- In almost all CD43/B220 FACS plots, the numbers are covering the cell populations in the Figures making it difficult to evaluate the data.

Sorry about the inconvenience. We have updated the figures.

- Minor point: The abbreviation for differentially expressed genes (DEGs) is give as acronym on page 11 and explained on page 14.

Thank you for pointing out that. We have updated them in the manuscript (line 270).

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Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my questions.

We thank the reviewer's approval of our revision.

Reviewer #2 (Remarks to the Author):

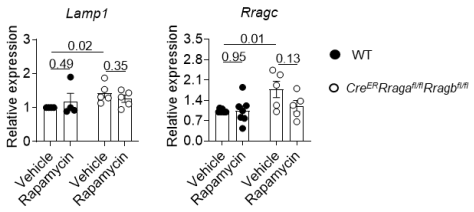
The authors made a good effort to experimentally address the comments from the reviewers. However, there are some concerns regarding interpretation of the data that they should address prior to publication:

We thank the reviewer for his/her appreciation of our effort.

(1) Just because the authors see further inhibition of IgG1 expression with Rapamycin treatment (Figure S1F), does not mean that RagA/RagB depletion is acting independently of mTORC1. If mTORC1 inhibition is incomplete following RagA/RagB deletion, the phenotypes may be further altered by Rapamycin. Similarly, because constitutively active TFEB may drive increased mTORC1 activation (Figure S6E), this suggests that the minimal reduction of mTORC1following RagA/B deletion could be due to increased nuclear TFEB and non-canonical activation of mTORC1. Finally, the rapamycin treatment studies (Figure 1G) are difficult to interpret. While it is true that rapamycin inhibits mTORC1, rapamycin has also been shown to paradoxically decrease phosphorylation of TFEB, resulting in nuclear exclusion (see PMID: 38195686). Hence, the changes seen following Rapa treatment of RagA/B deficient B cells are difficult to interpret.

We would like to clarify and reiterate several lines of evidence in support of our hypotheses.

1. Our data demonstrated that RagA/RagB deficient B cells have largely normal canonical mTORC1 activity (Fig.1C, 1D, 3F).
2. Constitutive active TFEB expression does not significantly increase mTORC1 activity (Fig. S6E). The reviewer correctly noticed that there was a trend of increased p-S6 and p-4EBP1 (but not statistically significant), however, this slight increase of mTORC1 was on the background of **RagA/RagB sufficient WT B cells**.
3. Regarding the potential of rapamycin affecting TFEB activity, the paper (PMID: 38195686) showed that rapamycin increases TFEB phosphorylation/activity only in the background of TSC2 deficiency, but not in the control Hela cells. Thus, this effect is context specific.
4. A recent publication (PMID: 39138218) demonstrated that rapamycin does not affect TFEB nuclear localization in primary B cells (Fig. 4I and 4J). Thus, it is unlikely that rapamycin affect TFEB transcriptional activity in murine B cells.
5. We have measured the expression of key TFEB target genes, *Lamp1* and *Rragc*, in rapamycin treated WT and RagA/RagB deficient B cells. Our data indicate that rapamycin does not substantially affect their expression in either WT or RagA/RagB KO B cells. Therefore, our data is consistent with the published literature (PMID: 39138218) that rapamycin does not affect TFEB activity.



6. We have demonstrated that TFEB overactivation negatively regulates TMRM and MTDR (Fig 6D, 6I and 6J) in primary B cells. Yet, rapamycin treatment did not further change the TMRM and MTDR levels in either WT or RagA/RagB deficient B cells (Fig. S4A and S4B). These data are consistent with our hypothesis that mTORC1 inhibition (either through rapamycin treatment or *Raptor* deletion, (Fig 5I, Fig. S4H)) does not affect TFEB activity and mitochondrial membrane potential.

Altogether, data from our manuscript and published literature support that rapamycin remains a valid tool to target mTORC1, at least in murine B cells.

(2) The authors appear to ignore the comments from reviewer 1 and 2 regarding the already documented roles of FLCN/Fnip1 in regulating phosphorylation of TFEB in response to RagGTPases (for example, see PMID: 32612235). In particular, In FLCN deficient cells, RagC (and mTORC1) is unable to bind and phosphorylate TFEB resulting in nuclear location and constitutive activation similar to RagA/B deficient cells. In addition, FLCN is also largely dispensible for phosphorylation of S6K and 4E-BP1 by mTORC1 (also similar to RagA/B deficient cells). Given the important roles of Fnip1/FLCN in B cell development and activation in response to nutrients, it is quite possible that the mechanism of TFEB constitutive nuclear localization in Rag dKO mice is due to improper regulation of the FLCN/Fnip1 pathway. This should be mentioned in the discussion. It is not surprising that the phenotypes of FLCN and Rag dKO mice are not identical, because they have both proteins likely have overlapping and independent functions.

We agree with the reviewer that the role of Fnip1/FLCN in the context of RagA/RagB deficiency warrants further investigation. Our manuscript demonstrated that dysregulation of TFEB mediated mitochondrial metabolism is responsible for many of the defects found in RagA/RagB deficient B cells. It is possible that abnormal Fnip1/FLCN might be one of the contributors connecting RagA/RagB deficiency and TFEB overactivation. Indeed, FLCN is also known to promote the expression of RagC and RagD. The complex interplays between Rag-GTPase, Fnip1/FLCN, TFEB/TFE3, and mTORC1 easily exceed the scope of our current manuscript. We have included a few sentences in this regard in the Discussion section (lines 478-481). Thank you!

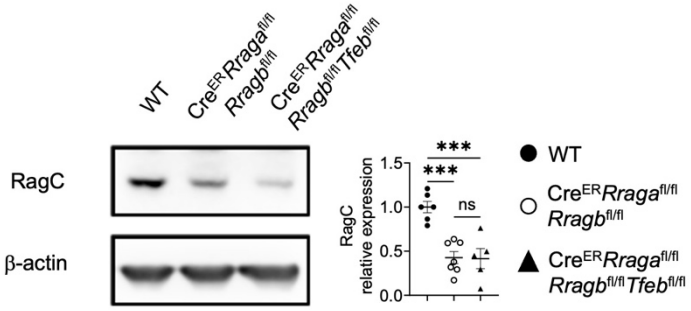
(3) Figure 1- font way too small to read

We have changed the image size in Figure 1.

(4) The question regarding if RagC/D is deleted in RagA/B deficient B cells, does this prevent mTORC1 activation was not really answered. Although published models suggest that full activation of Rag GTPase requires both RagA/RagB-GTP and RagC/RagD-GDP, their data suggest that RagA/B KO B cells have normal p-4EBP1 and reduced p-S6K and p-S6R. In addition, transcriptional activation of RagD has been shown to drive mTORC1 activation and cancer growth (PMID: 28619945, PMID: 31225432, PMID: 35654731)

We believe that a few lines of evidence support our conclusion that RagA/RagB deficiency abrogates Rag-GTPase complex.

1. **First and foremost**, biochemistry studies have long established that RagA and RagB stabilize RagC and RagD by forming heterodimers. Loss of RagA and RagB leads to the loss of RagC and RagD and vice versa on the protein level (PMID: 18497260, PMID: 25567907, PMID: 11073942), consequently genetic deletion of RagA/RagB has been extensively used to model Rag-GTPase deficiency in many different systems including immune cells (PMID: 31668641, PMID: 37380769). Indeed, RagC protein expression is also significantly and greatly reduced in RagA/RagB KO B cells, which is not affected by *Tfeb* deletion. Therefore, even though the transcription of RagC and RagD is increased in the absence of RagA/RagB, the protein expression of RagC, and possibly RagD, is greatly reduced and thus it is highly unlikely that any functional Rag-GTPase exist in RagA/RagB deficient B cells.



2. RagA/RagB deficient B cells do not have overtly overactivation of mTORC1, suggesting that the residual RagC and RagD proteins cannot promote mTORC1 without RagA/RagB (consistent with the current model).
3. TFEB deletion significantly restored RagC and RagD mRNA expression, but not RagC protein expression, in RagA/RagB deficient B cells (Fig. S6L). Yet, it did not significantly affect mTORC1 activity (Fig. S6M). Thus, the mRNA expression changes of RagC and RagD are not associated with mTORC1 activity change, consistent with the current model that RagC and RagD cannot stimulate mTORC1 independent of RagA/RagB.
4. Previous literature has indicated that mTORC1 can be activated independent of Rag-GTPase complex (PMID: 25567907, PMID: 24980141, PMID: 26774477). Thus, the largely normal mTORC1 activity in RagA/RagB KO B cells is most likely maintained by a Rag-GTPase complex independent mechanism.
5. The reviewer is correct that RagD (and RagC) mutations or variants can drive mTORC1 activation. However, these events always occur in the presence of normal RagA/RagB. We

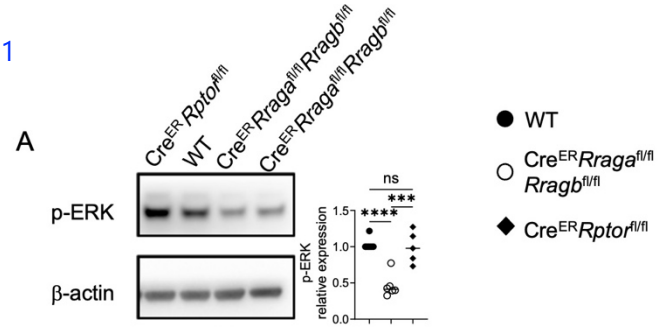
are not aware of any scenarios that RagD or RagC can promote mTORC1 in the absence of RagA/RagB.

(5) Reference 1 in the rebuttal is incorrect. I believe it should be PMID: 31676673
We apologize for the mistake. It should be PMID: 31676673. Thank you!

(6) In Rebuttal Figure 1A, what do the symbols refer to in the graph (left)? Need legend

We have added the legend.

Rebuttal Figure 1



(7) Lines 39-41 in the abstract " ….our data establish …. As an mTORC1 independent mechanism to coordinating nutrient sensing and mitochondrial metabolism" is overstated.

We have modified our language to reflect a degree of uncertainty throughout the manuscript regarding this point (lines 39, 98, 138, 177, 231, 432). Thank you!

(8) Line 81 "cytokine" should be "cytokines".

Thank you for pointing out the mistake. We have corrected it.

(9) Lines 97-99: I believe "independent of mTORC1" is overstated. Similar comments regarding line 426

We have modified our language to reflect a degree of uncertainty throughout the manuscript regarding this point (lines 39, 98, 138, 177, 231, 432).

Reviewer #3 (Remarks to the Author):

The authors addressed the previous concerns and questions adequately. With the new experiments and modifications, the clarity and robustness of the findings is greatly enhanced. Based on these extensive revisions, I believe the manuscript is now significantly improved and acceptable for publication.

We appreciate the reviewer's endorsement of our manuscript.