Peer Review File

Manuscript Title: SAR1B senses leucine levels to regulate mTORC1 signalling

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Ref #1

Chen et al identified SAR1B as a leucine sensor that modulates mTORC1 activity. Using siRNA screen combined with immunoprecipitation of GATOR2 to isolate GATOR2-interating proteins, they identified SAR1B by mass spec. Several lines of evidence were presented to support their conclusion. First, by knockdown of SAR1B, they showed that SAR1B is required to prevent mTORC1 activation in the absence of leucine but not other amino acids including other essential amino acids. The effect of SAR1B deficiency on mTORC1 activation was abolished when Rag-DN was co-expressed, suggesting that SAR1B is upstream of Rag activation. The interaction of Flag-SAR1B with GATOR2 components occurred under leucine-deprived conditions. Furthermore, among the GATOR2 components, SAR1B specifically interacts with Mios and they identified three key residues in SAR1B that are crucial for the interaction. Mios depletion abrogated the SAR1B deficiency-induced mTORC1 activation. The interaction of Flag-GATOR2 with SAR1B was abolished as leucine concentration increased. They also showed that SAR1B binds leucine (with a Kd of 2.1 uM) but not arginine and leucine binding caused a conformational change in SAR1B. Since Sestrin2 has been shown to be a leucine sensor, they further explored the relationship between these two sensors and found that SAR1B has more sensitivity to leucine and that they bind to different components of GATOR2. To further support the role of SAR1B in modulating mTORC1 signaling, they analyzed its role in C. elegans and lung cancer. In C. elegans they found that sar-1 also regulate TORC1 signaling and prevents the starvation-induced lifespan increase. Analysis of TCGA database revealed that SAR1B is frequently deleted in lung cancer. Silencing of SAR1A/B increased mTORC1 signaling and anchorage-independent growth as well as xenograft tumor growth and these tumors were sensitive to rapamycin.

Overall, the studies are interesting and support a role for SAR1B in modulating mTORC1 signaling in response to leucine. Figures 1 and 2 address possible mechanisms involved in SAR1B regulation of mTORC1 signaling while Figures 3 and 4 provide in vivo support and physiological relevance of SAR1B in mTORC1 signaling. The effects of SAR1B depletion on mTORC1 activation are robust and convincing. While the binding of leucine to SAR1B is also supported by their in vitro biochemical data, more interrogation is needed in order to support the conclusion that SAR1B senses leucine and that this occurs via regulation of GATOR2.

1. The interaction studies were mostly done on exogenous proteins. Do endogenous levels of SAR1B and GATOR2 display interaction in a leucine-sensitive manner?
2. An outstanding question is how the rise in leucine levels affects the SAR1B/GATOR2 interaction. The authors did a nice analysis on the binding of SAR1B to Mios. Is the SAR1B region binding to GATOR2 also the same region binding to Leucine? If not, does the leucine binding then induce conformational change that dissociates it from GATOR2? So far the evidence shown are highly correlative, mostly in vitro data. Extended Fig 3c showing SAR1B dissociation from lysosomes upon leu addition should be analyzed for GATOR2 and included in the main figure. Likewise, Fig 2f that analyzes effects of SAR1B mutants on lysosome localization should be quantitated and included in the main figure.
3. Do the SAR1B mutants that do not bind GATOR2 bind leucine? Are there SAR1B mutants that cannot bind leucine and will they constitutively bind GATOR2 and prevent mTORC1 activation even in the presence of leucine?

4. SAR1B deficiency leads to constitutive mTORC1 activation even in the absence of leucine; how does this affect autophagy? Would the SAR1B deficiency promote autophagy which could salvage leucine leading to mTORC1 activation? Or would the constitutive mTORC1 activation prevent autophagy?

5. Fig 2d: Does SAR1B interact with other GATOR2 components in vivo when Mios is depleted?

6. Fig 2g: Does the dissociation of SAR1B and GATOR2 also correspond to S6K1 phosphorylation?

7. In the analysis of SAR1A/B deficiency in lung cancer datasets, are the deficiencies linked to mTORC1 activation and/or other mutations that enhance mTORC1 activation eg TSC deletion?

The presentation of data should be improved. Some supporting data, particularly negative results should be moved to Extended Data and critical data should instead be included in the main body. For example:
In Figure 1, the data on other amino acids (Fig 1D) can be moved to supplementary. Fig 1h, 1i, ij as well. They all present negative results.
In Figure 2, Fig 2b can also be moved to supplementary.

Ref #2

In the manuscript titled "SAR1B senses leucine levels to regulate mTORC1 signaling", Chen et.al provide evidence that SAR1A/B senses leucine levels to regulate mTORC1 signaling by directly interacting with GATOR2. SAR1A/B prevents lysosomal recruitment of mTOR upon leucine deprivation through suppression of the RagA/B pathway. Furthermore, overexpression of SAR1B suppresses mTORC1 and promotes lifespan extension of C.elegans whereas down-regulation of SAR1A/B promotes mTORC1 activity and therefore facilitates cancer cell growth in vivo. The authors also examine the relationship of SAR1A/B and Sestrin2 with regards to their biochemical properties and potential cell type dependent expression and contribution to Leu-dependent mTORC1 regulation. The observations described in the report are interesting and important, however, there are several concerns that should be addressed.

Comments and concerns:

1. In figure 1d, siRNA to SAR1B does not result in increased mTORC1 signalling unlike the data in figures 1b, c, g, h, j and in several other figures. Thus, the data in 1d cannot be interpreted as the SAR1B knockdown did not activate mTORC1 above the control siRNA.

2. In figure 1j, rescue of siSAR1B with WT, SAR1B-GDP and SAR1b-GTP yielded similar results in that they all suppressed the activation of mTORC1 signaling by Leu. This experiment needs to be completed with SAR1A and SAR1B double knockdown or knockout +/- rescue experiments with WT, SAR1A-GDP or SAR1B-GDP, and SAR1A-GTP or SAR1B-GTP. These experiments will also help to address the conclusion by the authors that SAR1A and SAR1B are redundant.

3. Additionally, can the authors demonstrate that like SAR1B, is SAR1A binding to GATOR2 is also sensitive to Leu starvation conditions?

4. How is the interaction between SAR1B and Mios regulated by Leu?

5. Can the authors confirm that the status of lysosome association by the H58A, E114A and E122A SAR1B mutants?

6. What is the evidence that Leu binds to and directly affects SAR1B association with lysosomes

and GATOR2 ? Also does leu bind to SAR1A?

7. The authors propose that the effective concentration of Leu to dissociate SAR1B vs Sestrin2 from GATOR2 is approximately 10-fold different. It would be valuable to determine the IC50 of leucine activation of mTORC1 signaling in HEK293T cells with either SAR1B (and SAR1A) knockdown versus Sestrin2 (and sestrin1) knockdown to provide support that the in vitro binding data is relevant in cells.

8. Is C.elegans Sar-1 expressed throughout the organism or is there tissue-specific expression? What about ceSestrin expression?

9. Can the authors address whether Sar-1 or ceSestrin is the major Leu sensor in C.elegans?

10. The authors monitor ce-mTORC1 activation by monitoring nuclear localization of HLH-30 (ceTFEB). Nuclear localization of HLH-30 is not an accurate indicator of mTORC1 activity. TFEB can be phosphorylated by multiple kinases in addition to mTORC1 such as GSK3, ERK and PKC. In addition, Brugarolas and co-workers have reported that mTORC1 inhibitor rapamycin promotes nuclear exclusion of TFEB, in contrast to nuclear accumulation. S6K and rpS6 are conserved in C.elegans and therefore should be used to indicate TORC1 activity.

11. Similarly, please address the role of Sestrins in the lung cancer models used.

Ref #3

mTOR (for 'mechanistic target of rapamycin') kinase-mediated signalling regulates cell growth and proliferation in response to nutrient availability – particularly intracellular amino acid levels – and hormone-dependent mitogenic factors via promoting macromolecule synthetic processes such as translation and downregulating catabolic processes such as autophagy. Defects in this signalling system have been linked to changes in the rate of ageing, and implicated in various human pathologies including cancer. In this manuscript entitled 'SAR1B senses leucine levels to regulate mTORC1 signaling' the authors uncovered a novel, upstream regulatory mechanism of mTOR signalling, which has a huge potential significance in the fields of cell biology, genetics and medicine (general interest). They showed that SAR1B protein (a small GTPase) acting as a leucine sensor inhibits mTORC1 (the mTOR complex 1) signalling in an evolutionarily conserved manner (in both human cells and nematodes). Using IP and MS assays, SAR1B was demonstrated to physically bind the GATOR2 (but not GATOR1) complex, especially one of its subunit, Mios, to interfere with mTORC1 activity under leucine deficient conditions. The molecular background by which SAR1B-Mios interaction is established was nicely explored in the study. SAR1B known as a component of COPII (coat protein complex II) participating in ER-to-Golgi trafficking was identified as the only COPII subunit that interacts with GATOR2. Thus, the protein was identified to influence mTORC1 activity through a function that is independent of the COPII complex. Furthermore, SAR1B was also shown to interact with leucine, resulting in its dissociation from GATOR2 and a subsequent activation of mTORC1. When SAR1B dissociated from GATOR2 upon leucine availability, mTORC1 was translocated to the lysosomal surface for activation. As shown by the authors, SAR1B deficiency made cells insensitive to leucine starvation and constitutively activated mTORC1.
A few years ago, Sestrin2, an evolutionarily conserved stress-inducible protein, was identified as a leucine-sensing mTORC1 inhibitor (Wolfson, R. L. et al. Science 351:43-48, 2016 – received 570 citations). A great advance of the manuscript is that the authors clarified how SAR1B and Sestrin2 (and Sestrin1) are related to each other in leucine sensing and mTORC1 activation; according to the results, the proteins do not interact with each other, bind to different GATOR2 subunits, detect different structural features of leucine, sense different concentrations of leucine, and accumulate in distinct tissues to regulate mTORC1 signalling.
Finally, the authors showed that the SAR1B-GATOR2-mTORC1 signalling axis regulates lifespan in the nematode Caenorhabditis elegans, and SAR1b, together with its paralogue, SAR1A, inhibits

mTORC1-dependent tumour growth in mice.
The manuscript presents several novel data with significant basic science and medical implications. The material reflects a well-established work (solid experimental data and conclusions are well supported) that is basically suitable for publication in Nature. Before accepting the material for publication, the authors must address certain issues listed below.
Major concerns
1, The authors should test whether SAR1B activity is influenced by insulin/IGF1 signalling, a major regulator of nutrient stress response (as a complex interplay exists between insulin/IGF1 and mTORC1 signalling and the FOXO-Sestrin-AMPK-TORC1 axis is conserved in mammalian cells).
2, In the nematode-related part, it would be crucial to perform an epistasis analysis to prove that SAR-1 acts upstream of CeTOR/LET-363 in lifespan determination. CeTor(RNAi) animals are long-lived (PMID: 14668850), and this phenotype should not be suppressed in the sar-1(-/+) genetic background. CeTor-specific dsRNA should be injected into animals or, alternatively, the authors should use soaking in order to generate a starving condition. Why CeMios RNAi treatment had no effect on lifespan under starvation is not clear (SAR1B and Mios interact with each other in human cells).
3, SAR1A does not compensate for SAR1B deficiency in lung cancers; rather, SAR1A and SAR1B are simultaneously silenced. Why and how (in cultured cells there is a compensatory mechanism as the author showed)?
Minor points
1, English should be adopted to Nature (e.g., 'signaling' should be changed to signalling)
2, Figure legends are frequently not informative enough as they describe the experiment rather than reflecting the corresponding result. For example, Fig. 2h: 'Purification of recombinant proteins from HEK293T cells.' Here I would suggest 'Purification of recombinant proteins from HEK293T cells indicates that leucine can bind to SAR1B.'
3, As the authors stated, results uncover a two-phase mTORC1 activation by sequentially triggering dissociation of SAR1B and Sestrin2 from GATOR2. The biological relevance of this finding should be discussed.

Author Rebuttals to Initial Comments:

Response to Reviewers:

We thank all the reviewers for the positive and encouraging remarks on our work. We are particularly grateful for the constructive comments that have helped us to improve the study.

Referee #1 (Remarks to the Author):

Chen et al identified SAR1B as a leucine sensor that modulates mTORC1 activity. Using siRNA screen combined with immunoprecipitation of GATOR2 to isolate GATOR2-interating proteins, they identified SAR1B by mass spec. Several lines of evidence were presented to support their conclusion. First, by knockdown of SAR1B, they showed that SAR1B is required to prevent mTORC1 activation in the absence of leucine but not other amino acids including other essential amino acids. The effect of SAR1B deficiency on

mTORC1 activation was abolished when Rag-DN was co-expressed, suggesting that SAR1B is upstream of Rag activation. The interaction of Flag-SAR1B with GATOR2 components occurred under leucine-deprived conditions. Furthermore, among the GATOR2 components, SAR1B specifically interacts with Mios and they identified three key residues in SAR1B that are crucial for the interaction. Mios depletion abrogated the SAR1B deficiency-induced mTORC1 activation. The interaction of Flag-GATOR2 with SAR1B was abolished as leucine concentration increased. They also showed that SAR1B binds leucine (with a Kd of 2.1 uM) but not arginine and leucine binding caused a conformational change in SAR1B. Since Sestrin2 has been shown to be a leucine sensor, they further explored the relationship between these two sensors and found that SAR1B has more sensitivity to leucine and that they bind to different components of GATOR2. To further support the role of SAR1B in modulating mTORC1 signaling, they analyzed its role in C. elegans and lung cancer. In C. elegans they found that sar-1 also regulate TORC1 signaling and prevents the starvation-induced lifespan increase. Analysis of TCGA database revealed that SAR1B is frequently deleted in lung cancer. Silencing of SAR1A/B increased mTORC1 signaling and anchorage-independent growth as well as xenograft tumor growth and these tumors were sensitive to rapamycin.

Overall, the studies are interesting and support a role for SAR1B in modulating mTORC1 signaling in response to leucine. Figures 1 and 2 address possible mechanisms involved in SAR1B regulation of mTORC1 signaling while Figures 3 and 4 provide in vivo support and physiological relevance of SAR1B in mTORC1 signaling. The effects of SAR1B depletion on mTORC1 activation are robust and convincing. While the binding of leucine to SAR1B is also supported by their in vitro biochemical data, more interrogation is needed in order to support the conclusion that SAR1B senses leucine and that this occurs via regulation of GATOR2.

1. The interaction studies were mostly done on exogenous proteins. Do endogenous levels of SAR1B and GATOR2 display interaction in a leucine-sensitive manner?

Yes. Endogenous levels of SAR1B and GATOR2 also revealed a leucine-sensitive interaction (Extended Data Fig. 2f).

2. An outstanding question is how the rise in leucine levels affects the SAR1B/GATOR2 interaction. The authors did a nice analysis on the binding of SAR1B to Mios. Is the SAR1B region binding to GATOR2 also the same region binding to Leucine?

The leucine-binding region of SAR1B differs from the GATOR2-binding region, as the SAR1B mutants with no GATOR2-binding activity still bound leucine with similar affinities (Extended Data Fig. 4e, f).

If not, does the leucine binding then induce conformational change that dissociates it from GATOR2?

We thank the reviewer for the excellent question. To answer this question, in the revised manuscript, we first identified three potential leucine-binding sites in SAR1B by performing an alanine scan (individually mutating each amino acid residue of SAR1B to alanine, except the alanines themselves, which were mutated to aspartic acid) (Fig. 2e). We then showed that mutation of the leucine-binding sites prevented the conformational change of SAR1B upon leucine supplementation, as revealed by the circular dichroism spectroscopic analysis (Extended Data Fig. 4h). The mutations also induced a constitutive association of SAR1B with GATOR2 (Fig. 2f and Extended Data Fig. 4i). These results suggested that leucine binding induced a conformational change of SAR1B and dissociated it from GATOR2.

So far the evidence shown are highly correlative, mostly in vitro data. Extended Fig 3c showing SAR1B dissociation from lysosomes upon leu addition should be analyzed for GATOR2 and included in the main figure.

We have followed the reviewer's advice to analyze the lysosomal localization of GATOR2, together with SAR1B, in response to leucine addition and we have included the data in the main figure (Fig. 1i).

Likewise, Fig 2f that analyzes effects of SAR1B mutants on lysosome localization should be quantitated and included in the main figure.

We have followed the reviewer's advice to quantify the effects of SAR1B mutants (incapable of binding GATOR2) on lysosome localization of mTORC1. We included the results in the main figure (Fig. 1h).

3. Do the SAR1B mutants that do not bind GATOR2 bind leucine?

Yes. The SAR1B mutants that do not bind GATOR2 still bind leucine with similar binding affinities (Extended Data Fig. 4f).

Are there SAR1B mutants that cannot bind leucine and will they constitutively bind GATOR2 and prevent mTORC1 activation even in the presence of leucine?

We thank the reviewer for the excellent question. In the revised manuscript, we have performed a mutagenesis screen and identified three SAR1B mutants with reduced (V100A) or absent (A141D and E144A) leucine-binding activity (Fig. 2e). These three SAR1B mutants constitutively bind GATOR2 (Fig. 2f and Extended Data Fig. 4i) and prevent mTORC1 activation (Fig. 2g) even in the presence of leucine.

4. SAR1B deficiency leads to constitutive mTORC1 activation even in the absence of leucine; how does this affect autophagy? Would the SAR1B deficiency promote autophagy which could salvage leucine leading to mTORC1 activation? Or would the constitutive mTORC1 activation prevent autophagy?

We thank the reviewer for raising this question. Deficiency of SAR1B prevented leucine starvation-induced autophagy (Extended Data Fig. 1e). We further showed that autophagy was reactivated by administration of rapamycin to SAR1B-deficient cells, which suggests that mTORC1 acts upstream of autophagy and negatively regulates it (Extended Data Fig. 1f).

5. Fig 2d: Does SAR1B interact with other GATOR2 components in vivo when Mios is depleted?

To answer this question, we established a Mios knockout cell line. Endogenous SAR1B failed to interact with other GATOR2 components in the Mios knockout cells (Extended Data Fig. 2f, g).

6. Fig 2g: Does the dissociation of SAR1B and GATOR2 also correspond to S6K1 phosphorylation?

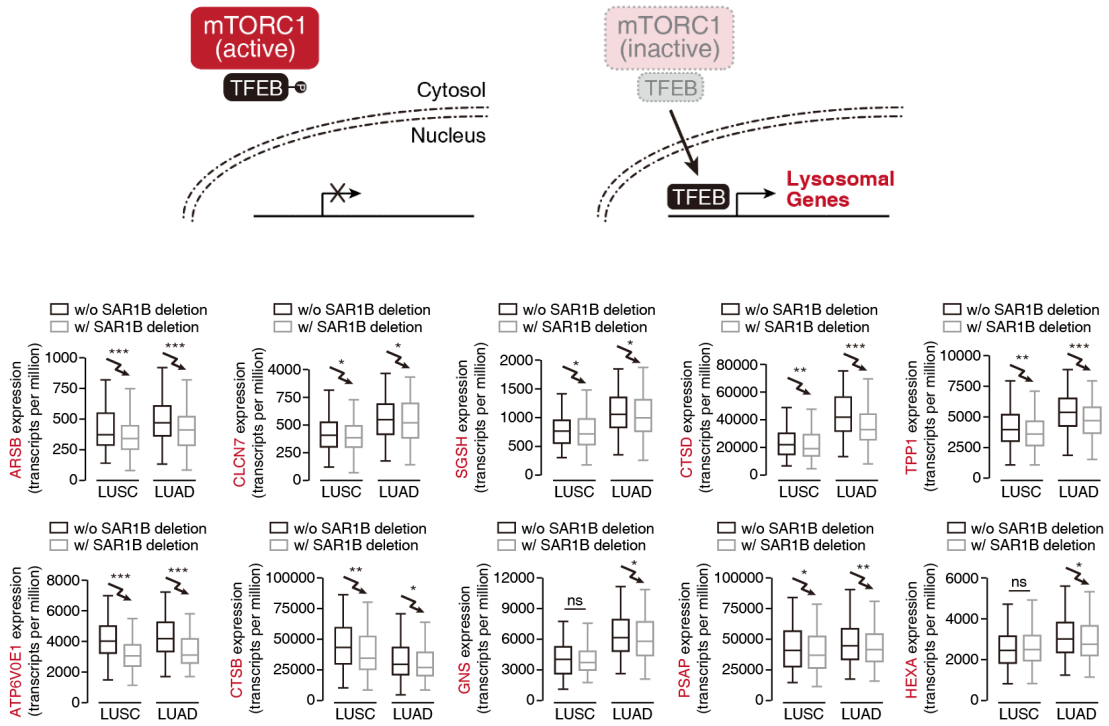
Yes. The dissociation of SAR1B and GATOR2 corresponds to S6K1 phosphorylation. Fig. 2g (now revised Extended Data Fig. 3b) was performed *in vitro* using purified recombinant proteins, so the S6K1 phosphorylation cannot be detected in this case. To answer the reviewer's question, we performed a similar experiment *in vivo* by stimulating cells with increasing concentrations of leucine. We found that the dissociation of SAR1B and GATOR2 corresponded to S6K1 phosphorylation (Extended Data Fig. 4m, n).

In addition, when SAR1B was constitutively associated with GATOR2 by mutating the leucine-binding sites in SAR1B, S6K1 was unable or less able to be phosphorylated (Fig. 2f, g). In contrast, when SAR1B and GATOR2 were constitutively dissociated by mutating the Mios-binding sites in SAR1B, S6K1 became constitutively phosphorylated (Fig. 1e, f).

7. In the analysis of SAR1A/B deficiency in lung cancer datasets, are the deficiencies linked to mTORC1 activation and/or other mutations that enhance mTORC1 activation eg TSC deletion?

To answer the reviewer's question, we obtained two human lung cancer cell lines with SAR1B copy loss and showed that mTORC1 was significantly activated in these two cell lines, compared to normal lung fibroblasts (Extended Data Fig. 8b).

In addition, because mTORC1 activation has been linked to the suppressed transcription of several lysosomal genes owing to the inactivation of transcription factor TFEB (Marco Sardiello et *al*., *Science* 2009; Agnes Roczniak-Ferguson et *al*., 2012, *Science Signaling*), we also analyzed the expression of 10 lysosomal genes controlled by TFEB. We found that the deficiency of SAR1A/B correlates with mTORC1 activation in human lung cancers. However, since Reviewer 2 raised some concerns about the relationship between TFEB and mTORC1, we did not provide this set of analyses in the revised manuscript.



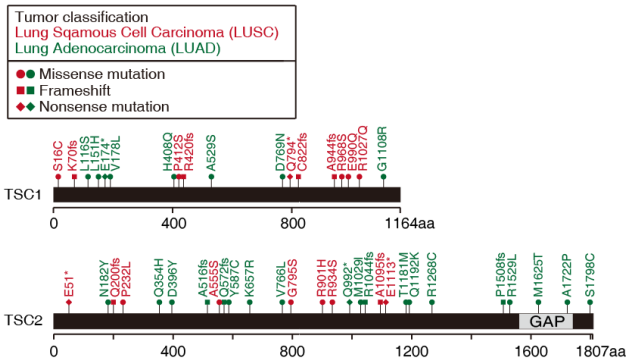
We have also followed the reviewer's advice to analyze TSC alterations in lung cancer datasets in TCGA. We found that there are limited cases of TSC alterations, including copy loss and gene mutations. Please see the results below. Due to the limited space, this result was not discussed in the revised manuscript.

Lung Squamous Cell Carcinoma (LUSC)
(469 samples)

Genes	Alterations		
	Homozygous deletion	Hemizygous deletion	Mutations
TSC1	2 (0.43%)	19 (4.05%)	10 (2.13%)
TSC2	0 (0.00%)	13 (2.77%)	17 (3.62%)

Lung Adenocarcinoma (LUAD)
(493 samples)

Genes	Alterations		
	Homozygous deletion	Hemizygous deletion	Mutation
TSC1	1 (0.20%)	11 (2.23%)	8 (1.62%)
TSC2	1 (0.20%)	9 (1.83%)	12 (2.43%)



The presentation of data should be improved. Some supporting data, particularly negative results should be moved to Extended Data and critical data should instead be included in the main body. For example:
In Figure 1, the data on other amino acids (Fig 1D) can be moved to supplementary. Fig 1h, 1i, ij as well. They all present negative results.
In Figure 2, Fig 2b can also be moved to supplementary.

We have followed the reviewer's advice to improve the data presentation by moving negative results to Extended Data and critical data to the main figures.

Referee #2 (Remarks to the Author):

In the manuscript titled "SAR1B senses leucine levels to regulate mTORC1 signaling", Chen et.al provide evidence that SAR1A/B senses leucine levels to regulate mTORC1 signaling by directly interacting with GATOR2. SAR1A/B prevents lysosomal recruitment of mTOR upon leucine deprivation through suppression of the RagA/B pathway. Furthermore, overexpression of SAR1B suppresses mTORC1 and promotes lifespan extension of C.elegans whereas down-regulation of SAR1A/B promotes mTORC1 activity and therefore facilitates cancer cell growth in vivo. The authors also examine the relationship of SAR1A/B and Sestrin2 with regards to their biochemical properties and potential cell type dependent expression and contribution to Leu-dependent mTORC1 regulation. The observations

described in the report are interesting and important, however, there are several concerns that should be addressed.

Comments and concerns:

1. In figure 1d, siRNA to SAR1B does not result in increased mTORC1 signalling unlike the data in figures 1b, c, g, h, j and in several other figures. Thus, the data in 1d cannot be interpreted as the SAR1B knockdown did not activate mTORC1 above the control siRNA.

We thank the reviewer for raising this question and guiding us to improve the clarity of the manuscript. In Fig. 1d (now revised Extended Data Fig. 1c), cells were treated with other amino acids (e.g., Val, Met, Phe, Lys, Arg, His), unlike in several other figures, in which cells were treated with leucine. SAR1B knockdown did not affect mTORC1 signalling when cells were treated with other amino acids, instead of leucine. These results indicate that SAR1B specifically responses to leucine signals to regulate mTORC1 signalling. According to Reviewer 1's advice, we moved the results of the other tested amino acids to the supplementary figures (Extended Data Fig. 1c).

2. In figure 1j, rescue of siSAR1B with WT, SAR1B-GDP and SAR1b-GTP yielded similar results in that they all suppressed the activation of mTORC1 signaling by Leu. This experiment needs to be completed with SAR1A and SAR1B double knockdown or knockout +/- rescue experiments with WT, SAR1A-GDP or SAR1B-GDP, and SAR1A-GTP or SAR1B-GTP. These experiments will also help to address the conclusion by the authors that SAR1A and SAR1B are redundant.

We performed the experiments suggested by the reviewer. Wild-type, GDP-locked and GTP-locked forms of SAR1A and SAR1B all restored the sensitivity of mTORC1 to leucine deficiency in SAR1A/B double knockdown cells (Extended Data Fig. 1o).

3. Additionally, can the authors demonstrate that like SAR1B, is SAR1A binding to GATOR2 is also sensitive to Leu starvation conditions?

Yes. SAR1A binds to GATOR2 under leucine-starved conditions and dissociates from GATOR2 upon leucine sufficiency when SAR1B is depleted (Extended Data Fig. 2d).

4. How is the interaction between SAR1B and Mios regulated by Leu?

We thank the reviewer for the excellent question. To answer this question, in the revised manuscript, we first identified three potential leucine-binding sites in SAR1B by performing an alanine scan (individually mutating each amino acid residue of SAR1B to alanine, except for alanines themselves, which were mutated to aspartic acid) (Fig. 2e). We then showed that mutation of these leucine-binding sites prevented the conformational change of SAR1B upon leucine supplementation, as revealed by the circular dichroism spectroscopic analysis (Extended Data Fig. 4h). The mutations also induced a constitutive association of SAR1B with Mios (Fig. 2f and Extended Data Fig. 4i). These results suggest that leucine regulates SAR1B and Mios interaction by directly binding to SAR1B, and inducing a conformational change of SAR1B.

5. Can the authors confirm that the status of lysosome association by the H58A, E114A and E122A SAR1B mutants?

The SAR1B mutants which cannot bind Mios (H58A, E114A and E122A) all failed to associate with lysosomes (Extended Data Fig. 3e). This result, together with the finding that GATOR2 constitutively docks on lysosomes regardless of leucine status (Fig. 1i), indicates that GATOR2 acts as a docking site on lysosomes, and SAR1B localizes to lysosomes by binding to GATOR2 upon leucine starvation.

6. What is the evidence that Leu binds to and directly affects SAR1B association with lysosomes and GATOR2?

We thank the reviewer for this great question. To address the question of whether leucine directly binds to SAR1B, we did a mutagenesis screen and identified three residues in SAR1B critical for leucine binding (V100, A141 and E144) (Fig. 2e). Next, we showed that mutation of the leucine-binding sites prevented the conformational change of SAR1B upon leucine supplementation, as revealed by the circular dichroism spectroscopic analysis (Extended Data Fig. 4h). Mutation of these sites also made SAR1B constitutively associated with lysosomes and GATOR2 (Fig. 2f and Extended Data Fig. 4i, j).

Also does leu bind to SAR1A?

Yes. SAR1A binds to leucine with an affinity similar to that of SAR1B (Extended Data Fig. 4c, d).

7. The authors propose that the effective concentration of Leu to dissociate SAR1B vs Sestrin2 from GATOR2 is approximately 10-fold different. It would be valuable to determine the IC50 of leucine activation of mTORC1 signaling in HEK293T cells with either SAR1B (and SAR1A) knockdown versus Sestrin2 (and sestrin1) knockdown to provide support that the in vitro binding data is relevant in cells.

We thank the reviewer for this excellent question. Unfortunately, we were unable to address this question by knockdown experiments, because knockdown of SAR1A/B or Sestrin1/2 made cells insensitive to leucine treatments and constitutively activated mTORC1 (supported by data in our study and data from Rachel L. Wolfson et *al*., 2016, *Science*).

To address this question in an alternative way, we used myotubes and adipocytes, in which different leucine sensor proteins are expressed. Adipocytes express Sestrin2 and myotubes express SAR1B and Sestrin1 (with similar leucine-sensing ability as Sestrin2) (Extended Data Fig. 6d). We treated adipocytes and myotubes with increasing concentrations of leucine and measured the IC50 of leucine that dissociates SAR1B or Sestrin1/2 from GATOR2 and activates mTORC1. In adipocytes, the IC50 of leucine that stimulates Sestrin2 dissociation and the corresponding mTORC1 activation was ~100 μM (Extended Data Fig. 6l, m). In myotubes, the IC50 of leucine for Sestrin1-dependent mTORC1 activation was similar to that for Sestrin2, while the IC50 of leucine for stimulating SAR1B dissociation and the corresponding mTORC1 activation was ~10 μM (Fig. 2i). Based on these results, the IC50 of leucine for stimulating SAR1B-dependent mTORC1 activation is 10-fold lower than the IC50 for stimulating Sestrin1/2-dependent mTORC1 activation in cells. This 10-fold difference is relevant to the *in vitro* data.

8. Is C.elegans Sar-1 expressed throughout the organism or is there tissue-specific expression? What about ceSestrin expression?

sar-1 (CeSAR1) is expressed in the intestine of *C. elegans*. *sesn-1* (CeSestrin) is expressed in the intestine and gonad (Extended Data Fig. 7i).

9. Can the authors address whether Sar-1 or ceSestrin is the major Leu sensor in C.elegans?

sar-1 is the major leucine sensor in *C. elegans* because knockdown of *sar-1* but not *sesn-1* significantly affected leucine sensing (Fig. 3g). Moreover, the sensing of arginine or isoleucine was not affected by *sar-1* knockdown (Extended Data Fig. 7 h).

10. The authors monitor ce-mTORC1 activation by monitoring nuclear localization of HLH-30 (ceTFEB). Nuclear localization of HLH-30 is not an accurate indicator of mTORC1 activity. TFEB can be phosphorylated by multiple kinases in addition to mTORC1 such as GSK3, ERK and PKC. In addition, Brugarolas and co-workers have reported that mTORC1 inhibitor rapamycin promotes nuclear exclusion of TFEB, in contrast to nuclear accumulation. S6K and rpS6 are conserved in C.elegans and therefore should be used to indicate TORC1 activity.

We followed the reviewer's advice to use phosphorylation of RSKS-1 (orthologue of human S6K1) as another indicator of TORC1 activity in *C. elegans*. The results are consistent with those reflected by HLH-30 nuclear localization (Fig. 3a-f).

11. Similarly, please address the role of Sestrins in the lung cancer models used.

We have followed the review's advice to test the role of Sestrins in the lung cancer models. We generated Sestrin1/2/3 triple knockout HFL1 cells and found that depletion of Sestrins also promoted tumor growth in subcutaneous and orthotopic cancer models (Extended Data Fig. 10f-i). This is consistent with the reports from other groups that Sestrin is downregulated in human lung cancers and implicated in lung cancer development (Kuan-Bing Chen et *al*., *Am J Transl Res* 2016; Boxiao Ding et *al*., *Cell Cycle* 2015).

Referee #3 (Remarks to the Author):

mTOR (for 'mechanistic target of rapamycin') kinase-mediated signalling regulates cell growth and proliferation in response to nutrient availability – particularly intracellular amino acid levels – and hormone-dependent mitogenic factors via promoting macromolecule synthetic processes such as translation and downregulating catabolic processes such as autophagy. Defects in this signalling system have been linked to changes in the rate of ageing, and implicated in various human pathologies including cancer. In this manuscript entitled 'SAR1B senses leucine levels to regulate mTORC1 signaling' the authors uncovered a novel, upstream regulatory mechanism of mTOR signalling, which has a huge potential significance in the fields of cell biology, genetics and medicine (general interest). They showed that SAR1B protein (a small GTPase) acting as a leucine sensor inhibits mTORC1 (the mTOR complex 1) signalling in an evolutionarily conserved manner (in both human cells and nematodes). Using IP and MS assays, SAR1B was demonstrated to physically bind the GATOR2 (but not GATOR1) complex, especially one of its subunit, Mios, to interfere with mTORC1 activity under leucine deficient conditions. The molecular background by which SAR1B-Mios interaction is established was nicely explored in the study. SAR1B

known as a component of COPII (coat protein complex II) participating in ER-to-Golgi trafficking was identified as the only COPII subunit that interacts with GATOR2. Thus, the protein was identified to influence mTORC1 activity through a function that is independent of the COPII complex. Furthermore, SAR1B was also shown to interact with leucine, resulting in its dissociation from GATOR2 and a subsequent activation of mTORC1. When SAR1B dissociated from GATOR2 upon leucine availability, mTORC1 was translocated to the lysosomal surface for activation. As shown by the authors, SAR1B deficiency made cells insensitive to leucine starvation and constitutively activated mTORC1.

A few years ago, Sestrin2, an evolutionarily conserved stress-inducible protein, was identified as a leucine-sensing mTORC1 inhibitor (Wolfson, R. L. et al. Science 351:43-48, 2016 – received 570 citations). A great advance of the manuscript is that the authors clarified how SAR1B and Sestrin2 (and Sestrin1) are related to each other in leucine sensing and mTORC1 activation; according to the results, the proteins do not interact with each other, bind to different GATOR2 subunits, detect different structural features of leucine, sense different concentrations of leucine, and accumulate in distinct tissues to regulate mTORC1 signalling.

Finally, the authors showed that the SAR1B-GATOR2-mTORC1 signalling axis regulates lifespan in the nematode Caenorhabditis elegans, and SAR1b, together with its paralogue, SAR1A, inhibits mTORC1-dependent tumour growth in mice.

The manuscript presents several novel data with significant basic science and medical implications. The material reflects a well-established work (solid experimental data and conclusions are well supported) that is basically suitable for publication in Nature. Before accepting the material for publication, the authors must address certain issues listed below.

Major concerns
1, The authors should test whether SAR1B activity is influenced by insulin/IGF1 signalling, a major regulator of nutrient stress response (as a complex interplay exists between insulin/IGF1 and mTORC1 signalling and the FOXO-Sestrin-AMPK-TORC1 axis is conserved in mammalian cells).

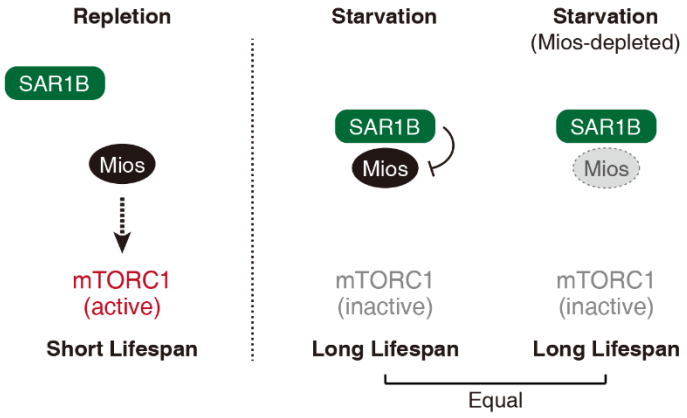
We have followed the reviewer's advice to test the role of SAR1B in insulin signalling. Knockdown of SAR1B did not affect insulin-dependent mTORC1 activation, which suggests that SAR1B is not involved in insulin signalling (Extended Data Fig. 1d).

2, In the nematode-related part, it would be crucial to perform an epistasis analysis to prove that SAR-1 acts upstream of CeTOR/LET-363 in lifespan determination. CeTor(RNAi) animals are long-lived (PMID: 14668850), and this phenotype should not be suppressed in the sar-1(-/+) genetic background. CeTor-specific dsRNA should be injected into animals or, alternatively, the authors should use soaking in order to generate a starving condition.

We have performed the experiment suggested by the reviewer using RNAi soaking. We confirmed that SAR-1 indeed acts upstream of CeTOR/LET-363 in *C. elegans* lifespan determination (Extended Data Fig. 7k).

Why CeMios RNAi treatment had no effect on lifespan under starvation is not clear (SAR1B and Mios interact with each other in human cells).

To better answer this question, we have drawn a schematic depicting the role of Mios (please see below). Mios is only active and functional under nutrient repletion. Upon starvation, Mios is inactivated by SAR1B, and its physical presence is dispensable. Thus, depletion of Mios has no impact on TORC1 activity and lifespan under nutrient-starved conditions.



3, SAR1A does not compensate for SAR1B deficiency in lung cancers; rather, SAR1A and SAR1B are simultaneously silenced. Why and how (in cultured cells there is a compensatory mechanism as the author showed)?

We thank the reviewer for raising this interesting question. In the revised manuscript, we employed a luciferase assay to show that the transcription of SAR1A was upregulated in HEK293T cells upon SAR1B deficiency, resulting in increased SAR1A mRNA and protein levels (Extended Data Fig. 1j-m). A similar transcriptional activation of SAR1A expression in lung fibroblasts was observed upon SAR1B deficiency (Extended Data Fig. 8a). In

contrast, in two non-small cell lung cancer cell lines with SAR1B copy loss, there was no obvious transcriptional activation of SAR1A upon SAR1B deficiency (Extended Data Fig. 8a). Collectively, these results suggest that in lung cancers, a transcriptional program that enhances SAR1A expression upon SAR1B deficiency might be silenced. The details of the mechanisms underlying these observations will be of great interest for future investigation.

Minor points
1, English should be adopted to Nature (e.g., 'signaling' should be changed to signalling)

We have followed the reviewer's advice to revise the manuscript.

2, Figure legends are frequently not informative enough as they describe the experiment rather than reflecting the corresponding result. For example, Fig. 2h: 'Purification of recombinant proteins from HEK293T cells.' Here I would suggest 'Purification of recombinant proteins from HEK293T cells indicates that leucine can bind to SAR1B.'

We have followed the reviewer's advice to revise the figure legends.

3, As the authors stated, results uncover a two-phase mTORC1 activation by sequentially triggering dissociation of SAR1B and Sestrin2 from GATOR2. The biological relevance of this finding should be discussed.

We thank the reviewer for the advice. In the revised manuscript, we presented a new set of experiments to show that in myotubes, where both SAR1B and Sestrin1 (with similar leucine-sensing ability as Sestrin2) are expressed, mTORC1 activity was regulated in a two-phase fashion due to sequential dissociation of SAR1B and Sestrin1 from GATOR2 in response to leucine signal (Fig. 2i). Based on these results, we have provided the following discussion for the biological relevance of these findings:

"Skeletal muscle mass constitutes ~40% of the body weight in humans. mTOR plays a crucial role in maintaining skeletal muscle mass by regulating the balance between protein synthesis and degradation. Notably, skeletal muscle cells are highly sensitive to changes in leucine levels, and leucine has been well documented as one of the most potent stimulators of skeletal muscle protein synthesis. The ability of SAR1B to sense a lower level of leucine may maintain basal mTOR activity and support a low level of protein synthesis, while the ability of Sestrin to detect accumulated leucine may induce a strong follow-up response. Therefore, SAR1B and Sestrin may be part of an elaborate signalling network in skeletal muscle to

precisely control leucine-dependent mTORC1 signalling and maintain muscle protein homeostasis."

Reviewer Reports on the First Revision:

Ref #1

In this revised submission, the authors provided more data to support that SAR1B senses leucine and that this regulation involves GATOR2. They identified leucine binding sites in SAR1B and performed mutagenesis analysis. These studies revealed that leucine binding induced a conformational change in SAR1B and dissociates it from GATOR2. The mutants that do not bind leucine constitutively bind GATOR2 and prevent mTORC1 activation. They also showed that deficiency in SAR1B prevented leucine starvation-induced autophagy.
The authors have fully addressed all my questions and comments.

Ref #2

The authors have done a nice job of addressing almost all of our previous concerns.

One remaining question is related to the immuno-staining in extended figure 3d. SAR1B was predominantly in the cytoplasm regardless of the presence of Leucine. However, upon isolation of lysosome, the authors observed enrichment of both SAR1B and GATOR2 in lysosomes which were dissociated by adding Leucine (extended figure 3e). Please discuss and provide an explanation for these observations.

Ref #3

The revised manuscript by Chen et al. has adequately addressed almost all of the concerns the referees raised. It now represents an excellent work reporting that the small GTPase SAR1B functions as a novel leucine sensor to modulate mTORC1 signalling. Results presented by this study certainly lead to a deeper insight into the mechanisms by which the mTORC1 pathway regulates cell growth and proliferation, and the balance between protein synthesis and degradation. Understanding better how intracellular leucine levels affect mTORC1 signalling has a significant medical implication. According to my standards, the material is now suitable for publication in Nature. Some minor points have remained only that can be easily fixed by a short polishing.
Minor comments
1, In the C. elegans part, when sens-1 is first mentioned, it would be important to add that the gene is orthologous to mammalian Sestrins, thereby acting as a leucine sensor.
2, The manuscript deals with mammalian and nematode systems. So, when discussing the role of mTORC1 signalling in ageing control, two relevant primary papers should be cited: PMID: 14668850 and PMID: 19587680.
3, Lifespan assays were performed with sar-1(+/-) heterozygous mutants because homozygous ones are unviable. The latter fact should be mentioned.
4, Polishing of the text should include: tumour instead of "tumor", and signalling instead of "signaling" (Fig. 4. legend)
5, At the beginning of the discussion section: " … sensor, Sestrin2, in four ways. 1) …" should be: "… sensor, Sestrin2, in four ways. First, … Second, …"

Author Rebuttals to First Revision:

Responses to reviewers:

Referee #1:

Remarks to the Author:

In this revised submission, the authors provided more data to support that SAR1B senses leucine and that this regulation involves GATOR2. They identified leucine binding sites in SAR1B and performed mutagenesis analysis. These studies revealed that leucine binding induced a conformational change in SAR1B and dissociates it from GATOR2. The mutants that do not bind leucine constitutively bind GATOR2 and prevent mTORC1 activation. They also showed that deficiency in SAR1B prevented leucine starvation-induced autophagy.

The authors have fully addressed all my questions and comments.

We thank the reviewer for the positive remarks.

Referee #2:

Remarks to the Author:

The authors have done a nice job of addressing almost all of our previous concerns.

One remaining question is related to the immuno-staining in extended figure 3d. SAR1B was predominantly in the cytoplasm regardless of the presence of Leucine. However, upon isolation of lysosome, the authors observed enrichment of both SAR1B and GATOR2 in lysosomes which were dissociated by adding Leucine (extended figure 3e). Please discuss and provide an explanation for these observations.

We have followed the reviewer's advice to provide an explanation for these observations.

"These results indicated that a small portion of SAR1B was localized on lysosomes to regulate mTORC1 signalling, while the vast majority of SAR1B was distributed throughout cells to control other biological processes such as vesicle formation."

Referee #3:

Remarks to the Author:

The revised manuscript by Chen et al. has adequately addressed almost all of the concerns the referees raised. It now represents an excellent work reporting that the small GTPase SAR1B functions as a novel leucine sensor to modulate mTORC1 signalling. Results presented by this study certainly lead to a deeper insight into the mechanisms by which the mTORC1 pathway regulates cell growth and proliferation, and the balance between protein synthesis and degradation. Understanding better how intracellular leucine levels affect mTORC1 signalling has a significant medical implication. According to my standards, the material is now suitable for publication in Nature. Some minor points have remained only that can be easily fixed by a short polishing.

Minor comments

1, In the C. elegans part, when sens-1 is first mentioned, it would be important to add that the gene is orthologous to mammalian Sestrins, thereby acting as a leucine sensor.

We have followed the reviewer's advice to add this notification.

2, The manuscript deals with mammalian and nematode systems. So, when discussing the role of mTORC1 signalling in ageing control, two relevant primary papers should be cited: PMID: 14668850 and PMID: 19587680.

We have followed the reviewer's advice to add these two references.

3, Lifespan assays were performed with sar-1(+/-) heterozygous mutants because homozygous ones are unviable. The latter fact should be mentioned.

We have followed the reviewer's advice to mention this fact.

4, Polishing of the text should include: tumour instead of "tumor", and signalling instead of "signaling" (Fig. 4. legend)

We have followed the *Nature* style and replaced “tumor” with “tumour” and “signaling” with “signalling”.

5, At the beginning of the discussion section: “ … sensor, Sestrin2, in four ways. 1) …” should be: “… sensor, Sestrin2, in four ways. First, … Second, …”

We followed the reviewer’s advice to edit the text.