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Methionine metabolism is essential for SIRT1-regulated mouse embryonic stem cell maintenance and embryonic development

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 April 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, the referees appreciate your analyses. However, much more in-depth insight and more thorough analyses are required to better support your claims.

Importantly,
- all technical concerns (referee #1, point 3; referee #3, first and fourth bullet point) need to be addressed
- the pluripotency data need to be solidified and lack of quantifications need to be addressed (referee #2, point 1; referee #3, third and last bullet point)
- changes in threonine metabolism in response to Sirt1 deficiency needs to be analyzed and put into the context of the existing literature (referee #1, point 1)
- rescue experiments need to be performed (referee #1, points 4, 6; referee #2, points 3, 4)

Note that an analysis of human ES/iPS cells is not needed.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers and especially those noted above. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised

version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

"Methionine metabolism is essential for SIRT1-regulated mouse embryonic stem cells maintenance and embryonic development" by Dr. Li and colleagues [EMBOJ-2017-96708]

General summary
The authors found that SIRT1 deficient ESCs displayed altered one-carbon metabolism. They then studied the relationship between Sirt1 and methionine metabolism and uncovered a novel role of SIRT for methionine metabolism during embryogenesis through regulating Mat2.
While the reviewer finds that the role of SIRT1 on methionine metabolism is potentially of interest, and the authors did overall very nice studies and provided solid data, there are several important issues to be addressed. For example, in mice, threonine metabolism is reported to provide a substantial fraction of the cellular glycine and acetyl-CoA for SAM synthesis (Wang et al, 2009; Shyh-Chang N et al, 2013). In the present manuscript, the authors did not deprive threonine from the medium. The authors should discuss the relationship of threonine and SIRT1 KO. There also arises the question whether SIRT1 has any role in hES/iPS cells.

Specific major concerns
1. Wang et al (2009) reported that mouse ESCs rely on Threonine metabolism. Shyh-Chang et al (2013) reported that Threonine metabolism works through SAM. The authors should show/ discuss the effects of SIRT KO on Threonine metabolism, and the effects in MD/MR conditions.
2. Figure 1: SIRT1 is expressed in mouse embryos and mouse ES cells. Is SIRT1 also expressed in humans ES/iPS cells?
3. Figure 2: The ESCs used for metabolism studies are cultured in presence of FBS. Serum contains amino acids. Why did the authors not use dialyzed serum? Is it because the KO ESCs cannot be maintained in dialyzed serum? If this is the case, the authors should provide discussions on this.
4. Figure 3: The title of the figure is misleading. The authors did not induce differentiation.
The authors just cultured the cells in ESC maintenance medium and found that the cells expressed several markers. Figure 3C: It is not clear how many hours (days) the cells are cultured under methionine restricted/deprived conditions. This should be noted in the legend. Also in material and methods, the timing of medium changes should be noted.
In this experiment, the authors did not initiate differentiation by culturing cells under differentiation condition. The cells did not differentiate but just lost their pluripotency and turned on some differentiation markers which are not normally expressed in undifferentiated ESCs.
Similarly, on page 7-8 the authors described that the ESCs are induced to differentiate under methionine depletion. This also should be rephrased. The ES cells might loose their potential as undifferentiated cells, which does not necessarily mean that the cells differentiated.
Another question is whether or not these changes are irreversible. Can SIRT1 KO ESCs after being treated with methionine deprivation/restriction but then switched back to normal medium reverse back to the pluripotent state?
5. Page 8: "Further analyses revealed that 24-hour methionine restriction followed by overnight methionine deprivation (labeled as MR/MD) induced significantly more apoptotic cells in SIRT1 deficient cells than in control mESCs (Fig. 3D and 3E)." Why did the authors not perform methionine deprivation for longer periods, but instead performing MR? Since threonine exists in the medium, cells might not become apoptotic easily. Did the authors also test the effects of threonine deprivation?
6. Figure 6E, G: The authors performed rescue experiments and overexpressed MAT2A in SIRT1 KO ESCs. Does the overexpression increase SAM concentration? How about rescuing by SAM addition instead of MAT2A overexpression?

Minor concerns
Figure 2C: figure should be reorganized so that the listed metabolites are coordinated with the heat maps, respectively.

Referee #2:

The manuscript by Li and coworkers presents a study by which SIRT1 regulates methionine metabolism in embryonic stem cells (mESCs).
The study presents several novel findings, including a potential role for SIRT1 in methionine metabolism and mESC maintenance. Several controls should be performed to solidify the authors' conclusions.

1. The precise role of SIRT1 in mESC maintenance needs clarification. The presented data seem preliminary and offer only a superficial survey. Figure 1 shows images of mESCs and staining. However, it is not clear these are actually different - can the authors provide quantification with statistical significance? Functionally, does SIRT1 affect mESC survival or differentiation into specific lineages? Further functional assays and markers would deepen these analyses.
2. The authors show convincingly that methionine and related metabolites are altered by SIRT1 loss. These studies were all steady state analysis - thus the pathways can only be inferred. They may want to show functionally whether Mat2a, Mat2b vs other enzymes in met metabolism are altered using tracing studies with isotopically labeled met. Alternatively - can the authors measure Mat2a/activity?
3. Can methionine or metabolites related to SAM rescue the mESCs phenotypes?
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6. The effect of methionine rescue of the SIRT1 KO embryos is difficult to interpret and can be due to numerous mechanisms unrelated to SAM.

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Tang et al. investigate the role of Sirt1 in the maintenance of mouse embryonic stem cells (ES cells). The authors provide experimental evidence that Sirt1 deletion changes methionine metabolism in ES cells leading to reduced production of SAMs required for the maintenance of histone methylation marks such as the activating mark H3K4-3me. The authors demonstrate that methionine deprivation limits ES cell maintenance accompanied by the induction of differentiation pathways and the suppression of self renewal pathways; Sirt1 deletion mimics this phenotype by inhibiting methionine metabolism and SAM formation. The authors demonstrate that Sirt1 regulates methionine metabolism and SAM formation via stabilizing c-myc and N-myc by de-acetylation. C-myc and N-myc in turn regulate the expression of Mat2 enzymes - key enzymes of SAM formation. Finally the authors provide evidence that the findings apply to early embryogenesis. Methionine depletion enhances death of Sirt1 deficient embryos. Vice versa, methionine pre-feeding enhances survival of early embryos and facilitates birth of Sirt1 deficient embryos.

Overall, the authors provide a compelling study on a new function of Sirt1 in regulating methionine metabolism and SAM formation in ES cells influencing epigenetic regulation of stemness and differentiation. The study widens our understanding of Sirt1 in stem cell biology and provides sound evidence that Sirt1 connects the energy state of ES cells towards methionine metabolism and epigenetic regulation of pluripotency and early embryonic development. I only have a few detail remarks on the study that should be addressed prior to publication:

- On the bottom of page 15 the authors conclude that the experiment on methionine deprivation in embryogenesis „indicated that methionine restriction selectively reduces the survival rate of SIRT1 KO embryos". This conclusion is not justified by the data. It is possible that methionine depletion also has a negative effect on Wt embryos but a more pronounced effect on Sirt1 KO embryos. The

authors should provide detailed numbers on litter size etc to make that conclusion more accurate.

- Figure Legend 5 should spell out what IPA analysis refers to.

- Figure 6B shows protein levels of MAT2a in Sirt1-KO and WT ES cells; the differences look not very significant under normal conditions (200μM methionine in medium), but become apparent under methionine restriction, yet methionine metabolism and SAM formation is already significantly reduced under normal conditions (200μM methionine) as shown in other figures. The authors should reconcile the experimental results and provide an explanation.

- The alkaline-phosphatase staining to illustrate ES cell pluripotency is fine, but I would like to see more quantitative data for some of the key experiments possibly on ES cell pluripotency employing for example FACS analysis of Oct4-reporter ES lines

- The Western Bolt on c-Myc-acetylation in Fig. 7G is not very convincing and should be repeated. In general, the Western Blots lack size markers. Full blots should be provided and statements on n-numbers included.

- The in vivo experiments on embryogenesis are very interesting but somehow puzzling: Fig. 8E shows that methionine restriction aggravates developmental defects at later stages of embryogenesis, 8F shows that pre-feeding of methionine can partially rescue developmental defects thereby facilitating the birth of Sirt1-KO mice. However, this rescue does not work when initiated from E0.5 onwards but only when prefeeding is started on females before fertilization. The authors conclude that methionine supplementation rescues defects in early embryogenesis and that this is required to get life pubs of Sirt1 KOs. Does that mean that the developmental defects at later stages of embryonic development are not lethal? Are those defects implemented during early stages of development inducing lethality by inducing other developmental defects and altered embryogenesis that cannot be rescued by methionine supplementation at later stages? I would assume that defects in early development would not lead to embryos that develop into later stages. The authors should explain this more carefully and if possible include a description on morphology and defects at early and late stages of embryogenesis and how these are rescued by methionine supplementation.

1st Revision - authors' response 24 July 2017

Referee #1:

"Methionine metabolism is essential for SIRT1-regulated mouse embryonic stem cells maintenance and embryonic development" by Dr. Li and colleagues [EMBOJ-2017-96708]

General summary

The authors found that SIRT1 deficient ESCs displayed altered one-carbon metabolism. They then studied the relationship between Sirt1 and methionine metabolism and uncovered a novel role of SIRT for methionine metabolism during embryogenesis through regulating Mat2.
While the reviewer finds that the role of SIRT1 on methionine metabolism is potentially of interest, and the authors did overall very nice studies and provided solid data, there are several important issues to be addressed. For example, in mice, threonine metabolism is reported to provide a substantial fraction of the cellular glycine and acetyl-CoA for SAM synthesis (Wang et al, 2009; Shyh-Chang N et al, 2013). In the present manuscript, the authors did not deprive threonine from the medium. The authors should discuss the relationship of threonine and SIRT1 KO. There also arises the question whether SIRT1 has any role in hES/iPS cells.

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We did not initially focus on threonine metabolism because this pathway was not significantly altered in KO mESCs cultured in complete medium (Figure EV2C and Table EV1). The key gene that mediates the impact of threonine on SAM production, Tdh, was also not significantly changed in KO mESCs compared to WT mESCs in all different conditions (please check our microarray dataset, GSE77757). Moreover, in the study by *Shyh-Chang et al.* (*Science*, 2013), Tdh mediated synthesis of SAM from threonine was found to only contribute about 10% of the cellular SAM pool. Therefore, most of the cellular SAM is still coming from methionine.

Per the reviewer's suggestion, we performed the threonine depletion experiment on WT and KO mESCs. Our new results in Figure EV3A (-Thr) indicated that threonine depletion induced massive cell death in both WT and KO mESCs to a comparable extent. This result is not surprising because threonine is required for the growth of mESCs through both SAM dependent and independent mechanisms (Wang et al., Science, 2009; Shyh-Chang et al., Science, 2013a).

2. Figure 1: SIRT1 is expressed in mouse embryos and mouse ES cells. Is SIRT1 also expressed in humans ES/iPS cells?

Yes, SIRT1 is also highly expressed in human ESCs by our microarray analysis and immunoblotting analysis. Another postdoctoral fellow in the lab is currently working to dissect its functions in these cells.

3. Figure 2: The ESCs used for metabolism studies are cultured in presence of FBS. Serum

contains amino acids. Why did the authors not use dialyzed serum? Is it because the KO ESCs cannot be maintained in dialyzed serum? If this is the case, the authors should provide discussions on this.

We did not use dialyzed serum due to concerns regarding the possible loss of growth factors or small molecule metabolites that are essential for maintenance of ESC pluripotency, particularly that of KO ESCs. Additionally, since we were primarily focused on methionine restriction induced loss of pluripotency instead of complete methionine depletion induced cell death, we feel the small amount methionine in the 10% serum will not significantly impact our results.

Per the reviewer's suggestion, we cultured WT and KO mESCs in the M10 medium containing 10% of dialyzed serum. Our new results in Figure R1 (at the end of this response) showed that when cultured in this medium, KO mESCs still displayed a reduced AP staining intensity compared to WT mESCs (Figure R1A), and both WT and KO mESCs underwent massive cell death upon methionine depletion (Figure R1B). The cell death of WT mESCs cultured in this medium was more severe than that in medium containing regular serum (Figure EV3A and EV5A), suggesting that the dialyzed serum is missing some components that are essential for mESC survival. Additionally, both WT and KO mESCs had a reduced AP staining intensity when cultured in the 10% dialyzed serum compared to normal serum, further supporting our original concerns.

4. Figure 3: The title of the figure is misleading. The authors did not induce differentiation. The authors just cultured the cells in ESC maintenance medium and found that the cells expressed several markers. Figure 3C: It is not clear how many hours (days) the cells are cultured under methionine restricted/deprived conditions. This should be noted in the legend. Also in material and methods, the timing of medium changes should be noted.
In this experiment, the authors did not initiate differentiation by culturing cells under differentiation condition. The cells did not differentiate but just lost their pluripotency and turned on some differentiation markers which are not normally expressed in undifferentiated ESCs. Similarly, on page 7-8 the authors described that the ESCs are induced to differentiate under methionine depletion. This also should be rephrased. The ES cells might loose their potential as undifferentiated cells, which does not necessarily mean that the cells differentiated.

The reviewer is correct that the SIRT1 deficient mESCs just started to express some differentiation markers when cultured in complete or methionine restricted medium. We changed the title of Figure 3 into "SIRT1 deficient mESCs are sensitive to methionine restriction induced **loss of pluripotency** and apoptosis". Thanks for the suggestion.

In Figure 3C, the cells were cultured as in Figure 3A, in the complete M10 medium or a M10 medium containing 6 μM methionine for 48 hours.

Another question is whether or not these changes are irreversible. Can SIRT1 KO ESCs after being treated with methionine deprivation/restriction but then switched back to normal medium reverse back to the pluripotent state?

To answer this question, we cultured WT and SIRT1 KO mESCs in M10 medium containing no methionine for 24 hours, followed by adding back 0, 6, 100, 200, and 400 μM methionine for additional 24 hours. As shown in new Figure EV6, adding back methionine switched both WT and KO mESCs back to a more pluripotent state (Figure EV6 A). Moreover, adding back methionine also dose-dependently reversed methionine-depletion induced apoptosis in KO mESCs (Figure EV6 B).

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As stated on Page 8, the reason why we did not perform methionine deprivation for a longer period, but instead performing MR is because we would like to focus on the involvement of SIRT1 in methionine mediated metabolic regulation of pluripotency maintenance but not on methionine-depletion induced stress responses and cell death. As shown in Figure EV3A, EV4A, and EV5A, although SIRT1 deficient mESCs appeared to be more differentiated upon methionine depletion compared to control mESCs, both control and SIRT1 deficient mESCs were experiencing massive cell death under this condition, making it difficult to dissect the direct role of SIRT1 in maintenance of the pluripotency of mESCs. We therefore titrated methionine concentration in the M10 medium to find an optimal methionine concentration range in which: (1) cell death from both WT and SIRT1 KO deficient mESCs were minimal (Figure EV5C); (2) the pluripotency difference between controls and SIRT1 deficient mESCs was the best (Figure EV5B, EV6A, and EV6B).

For the above reasons, we believe it is not necessary to assess the impact of threonine deprivation in our study because threonine is required for the growth of mESCs through both SAM dependent and independent mechanisms (Wang et al., Science, 2009; Shyh-Chang et al., Science, 2013a). As a result, threonine depletion leads to dramatic cell death of mESCs (Wang et al., Science, 2009; Shyh-Chang et al., Science, 2013a), which will further complicate our efforts to understand the role of SIRT1 in the maintenance of mESCs pluripotency. However, per the reviewer's request, we tested the impact of threonine deprivation on WT and SIRT1 KO mESCs. As expected (Figure EV3A), threonine depletion (-Thr) induced massive cell death in both WT and KO mESCs. Since the cell death was so severe, no difference was observed between WT and SIRT1 KO mESCs.

6. Figure 6E, G: The authors performed rescue experiments and overexpressed MAT2A in SIRT1 KO ESCs. Does the overexpression increase SAM concentration? How about rescuing by SAM addition instead of MAT2A overexpression?

Yes, overexpression of MAT2A increased cellular SAM concentration and rescued SAM deficiency of SIRT1 KO mESCs (Figure 6D, SAM).

We have spent a long time trying to rescue the SIRT1 KO phenotypes by adding SAM into the culture medium during our study. The lesson we learned is that mammalian cells do not have a SAM transporter on the plasma membrane, so SAM has a poor membrane permeability to mammalian cells, requiring about 20-fold membrane gradient to go into these cells. Given the average intracellular concentration of SAM is 50-150 μM as well as the fact that SAM is not stable and will degrade into various byproducts with half-life time around 12 hours at room temperature at neutral pH (personal communication with Dr. Minkui Luo from MSKCC), we needed to provide more than 500-1500 μM of SAM in medium in order to rescue the 40-50% of SAM reduction in SIRT1 KO mESCs. These high concentrations of SAM were toxic to mESCs in our hands. We further found out by LC-MS that the SAM concentrations that can be tolerant by mESCs in our hands (25-50 μM) did not significantly impact the intracellular SAM pool. For all these reasons, we decided that the best way to rescue the intracellular SAM pool is to overexpress MAT2A.

Minor concerns
Figure 2C: figure should be reorganized so that the listed metabolites are coordinated with the heat maps, respectively.

Thanks for pointing this out for us. We checked them to make sure the alignment works well in the revised version.

Referee #2:

The manuscript by Li and coworkers presents a study by which SIRT1 regulates methionine metabolism in embryonic stem cells (mESCs).

The study presents several novel findings, including a potential role for SIRT1 in methionine metabolism and mESC maintenance. Several controls should be performed to solidify the authors' conclusions.

1. The precise role of SIRT1 in mESC maintenance needs clarification. The presented data seem preliminary and offer only a superficial survey. Figure 1 shows images of mESCs and staining. However, it is not clear these are actually different - can the authors provide quantification with statistical significance? Functionally, does SIRT1 affect mESC survival or differentiation into specific lineages? Further functional assays and markers would deepen these analyses.

Thanks for the insightful suggestion. We performed a few additional experiments to quantify the difference between WT and SIRT1 KO mESCs during revision, including:

(1) Direct measurement of the activities of AP from total cell lysates of WT and KO mESCs, showing that SIRT1 KO mESCs have a 49% reduction of the AP activity compared to WT mESCs when cultured in complete M10 medium (Figure 1F).
(2) Immuno-blot analysis of two pluripotency markers, Nanog and Oct4, showing that SIRT1 KO mESCs display reduced expression of these two markers when cultured in complete M10 medium (Figure 1G).

(3) FACS analysis of Nanog expression, showing that SIRT1 KO mESCs have about 30% reduction of Nanog protein levels compared to WT mESCs when cultured in complete M10 medium (Figure 1H and EV1).

So, all these results support our conclusion that SIRT1 regulates the maintenance of pluripotent mESCs.

The functional impact of SIRT1 deficiency on mESC differentiation have been analyzed in one of our previous studies (Tang et al., 2014, Mol Cell 55: 843-55). Our results from that study indicated that SIRT1 deficiency enhances the expression of a number of differentiation markers from different linages upon treatment with retinoic acids (Figure 3 and 4 in Mol Cell 55: 843-55).

2. The authors show convincingly that methionine and related metabolites are altered by SIRT1 loss. These studies were all steady state analysis - thus the pathways can only be inferred. They may want to show functionally whether Mat2a, Mat2b vs other enzymes in met metabolism are altered using tracing studies with isotopically labeled met. Alternatively - can the authors measure Mat2a/activity?

Many thanks for the suggestion. We did not perform a tracing experiment because of two reasons: Firstly, the steady state levels of these metabolites are more relevant to final biological outputs than their fluxes. For example, Mentch et al. (Mentch et al., *Cell Metabolism*, 2015) have shown that the steady cellular SAM level and the SAM/SAH ratio can directly impact histone methylation levels/patterns and altering gene expression. Secondly, our lab/institute do not have the capacity to perform the tracing experiment. During our study, we have also consulted a couple of experts in the methionine field, they agreed with us that the tracing experiment is not essential for our conclusion.

Per the reviewer's request, we tried a few strategies to develop an assay for the direct measurement of the MAT2A activity. Attempts to measure the loss of methionine and the production of SAM were all failed due to lack of reliable methods to measure methionine and SAM without LC/MS. We eventually employed an enzyme coupled colorimetric detection method to indirectly measure the phosphate production from the MAT2A catalyzed reaction. The MAT2A activity was calculated by deducing A_{630nm} (-methionine) from A_{630nm} (+methionine). Although this is not a very clean assay due to interference of other phosphate-producing reactions from total cell lysates, data from three independent measurements were consistent and difference between WT and SIRT1KO was statistically significant. The result from these three experiments is now in Figure EV8C.

3. Can methionine or metabolites related to SAM rescue the mESCs phenotypes?

The complete M10 medium contains 200 μM methionine, which is already very high, so it is not easy to further supplement methionine in the medium to rescue the KO mESC phenotypes. However, as we showed in Figure 3, Figure EV3 and EV4, SIRT1 deficient mESCs have milder phenotypes in complete medium compared to the methionine restricted or methionine depleted medium, indicating that methionine supplementation alleviates the phenotypes of KO mESCs. Moreover, as stated in our response to reviewer #1, we cultured WT and SIRT1 KO mESCs in

M10 medium containing no methionine for 24 hours, followed by adding back 0, 6, 100, 200, and 400 μM methionine for additional 24 hours. Our data indicate that adding back methionine switched both WT and KO mESCs back to a more pluripotent state, also dose-dependently reversed methionine-depletion induced apoptosis in KO mESCs (Figure EV6).

Again as stated in our response to reviewer #1, we have tried to rescue the SIRT1 KO phenotypes by directly adding SAM into the culture medium during our study, we only learned that mammalian cells do not have a SAM transporter on the plasma membrane, so SAM has a poor membrane permeability to mammalian cells, requiring about 20-fold membrane gradient to go into these cells. Given the average intracellular concentration of SAM is 50-150 μM as well as the fact that SAM is not stable and will degrade into various byproducts with half-life time around 12 hours at room temperature at neutral pH (personal communication with Dr. Minkui Luo from MSKCC), we needed to provide more than 500-1500 μM of SAM in medium in order to rescue the 40-50% of SAM reduction in SIRT1 KO mESCs. These high concentrations of SAM were toxic to mESCs in our hands. We further found out by LC-MS that the SAM concentrations that can be tolerant by mESCs in our hands (25-50 μM) did not significantly impact intracellular SAM pool. For all these reasons, we decided that the best way to rescue the intracellular SAM pool is to overexpress MAT2A.

4. Does SIRT1 overexpression rescue phenotypes of mESCs, methionine and SAM? The critical control of an sh-insensitive clone of SIRT1 should be utilized in Figure 3.

Yes, SIRT1 overexpression rescued the protein levels of MAT2A (Figure 6C), AP staining intensity (Figure 3F), Nanog expression (Figure 3G), and MR/MD induced apoptosis in SIRT1 KO mESCs (Figure 3H).

5. Does SIRT1 directly interact with or deacetylate MAT2A to regulate its activity?

It is a great question, since MAT2A has been reported as an acetylated protein, and acetylation promotes its degradation (Yang et al., *Nature Communications*, 2015, 7973). However, we were unable to check the interaction between endogenous MAT2A and SIRT1 in mESCs by CoIP due to the similar size of MAT2A to the IgG heavy chain. Overexpressed HA-MAT2A appeared to interact with SIRT1 when pulled down with anti-HA beads (Figure R2 at the end of this response), but again we were not able to check its acetylation status due to the similar size of this protein to the IgG heavy chain.

Despite this observation, however, we did not believe the direct modification and interaction of MAT2A by SIRT1significantly contribute to the regulation of its expression/activity. Firstly, the reduced protein levels of endogenous MAT2A in SIRT1 KO mESCs were associated with reduced mRNA levels (Figure 6A), suggesting that transcriptional regulation is the major mechanism. Secondly, the exogenous MAT2A (HA-MAT2A) did not appear to be less stable in SIRT1 KO mESCs (Figure 6E)—the HA-MAT2A protein in fact was always higher in KO mESCs compared to WT mESCs, in spite of comparable mRNA levels in these two cells (Figure EV8B). More importantly, the high HA-MAT2A in SIRT1 KO mESCs was associated with high SAM levels compared to those in WT mESCs (Figure 6D), indicating that exogenous MAT2A is

fully functional in the absence of SIRT1. Therefore, SIRT1-dependent modification of MAT2A, if any, does not appear to significantly impact its activity.

6. The effect of methionine rescue of the SIRT1 KO embryos is difficult to interpret and can be due to numerous mechanisms unrelated to SAM.

We completely agree with the reviewer in this regard since methionine has a number of important functions in additional to SAM production. The cleanest rescue experiment is to directly supplement SAM. We have done the dietary SAM supplementation experiment and observed some rescue effects on the neonatal lethality of SIRT1 KO pups. However, due to its poor membrane permeability to mammalian cells and low stability (as outlined in response # 3), this effect is likely to arise from thiol and adenine byproducts according to experts in the field. Therefore, we were unable to get a clear answer with direct SAM supplementation either. We feel the methionine rescue experiment was the best one we can perform under the current circumstances. Future studies with a cell permeable SAM (when available) or transgenic mouse lines overexpressing Mat2a will help to directly test the importance of SAM production in SIRT1 deficiency induced development defects.

Referee #3:

Tang et al. investigate the role of Sirt1 in the maintenance of mouse embryonic stem cells (ES cells). The authors provide experimental evidence that Sirt1 deletion changes methionine metabolism in ES cells leading to reduced production of SAMs required for the maintenance of histone methylation marks such as the activating mark H3K4-3me. The authors demonstrate that methionine deprivation limits ES cell maintenance accompanied by the induction of differentiation pathways and the suppression of self renewal pathways; Sirt1 deletion mimics this phenotype by inhibiting methionine metabolism and SAM formation. The authors demonstrate that Sirt1 regulates methionine metabolism and SAM formation via stabilizing c-myc and N-myc by de-acetylation. C-myc and N-myc in turn regulate the expression of Mat2 enzymes - key enzymes of SAM formation. Finally the authors provide evidence that the findings apply to early embryogenesis. Methionine depletion enhances death of Sirt1 deficient embryos. Vice versa, methionine pre-feeding enhances survival of early embryos and facilitates birth of Sirt1 deficient embryos.

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The authors should provide detailed numbers on litter size etc to make that conclusion more accurate.

Thanks for this insightful suggestion. We in fact analyzed the litter size of our breeders under chow diet or MR diet for different times before finalizing our MR diet feeding procedure. As shown in Figure EV10B, MR diet feeding from E0.5 did not significantly affect the litter size, while MR diet prefeeding for 1 week significantly reduced the litter size. This is the reason why we used MR diet feeding from E0.5 as our final feeding protocol. Since the litter sizes were comparable between chow diet fed dams and MR diet fed dams, our data indicate that maternal methionine restriction (from E0.5) selectively reduces the survival rate of SIRT1 KO embryos.

- *Figure Legend 5 should spell out what IPA analysis refers to.*

Thanks, it is fixed.

- *Figure 6B shows protein levels of MAT2a in Sirt1-KO and WT ES cells; the differences look not very significant under normal conditions (200μM methionine in medium), but become apparent under methionine restriction, yet methionine metabolism and SAM formation is already significantly reduced under normal conditions (200μM methionine) as shown in other figures. The authors should reconcile the experimental results and provide an explanation.*

Thanks for pointing this out. The MAT2A protein levels were different in WT and KO mESCs when cultured in the complete medium, but the difference was always smaller than that in MR medium (just like the mRNA levels). That's why in some blots it was not obvious. We quantified the relative MAT2A protein levels under different conditions from 5 independent experiments (including the one in Figure 6B), and the results were shown Figure EV8B. Additionally, we measured the activity of MAT2A from total cell lysates, and the results also showed that MAT2A activity was significantly lower in OK mESCs when cultured in the complete medium (Figure EV8C).

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(1) Direct measurement of the activities of AP from total cell lysates of WT and KO mESCs, showing that SIRT1 KO mESCs have a 49% reduction of the AP activity compared to WT mESCs when cultured in complete M10 medium (Figure 1F).
(2) Immuno-blot analysis of two pluripotent markers, Nanog and Oct4, showing that SIRT1 KO mESCs display reduced expression of these two markers when cultured in complete M10 medium (Figure 1G).
(3) FACS analysis of Nanog expression, showing that SIRT1 KO mESCs have about 30% reduction of Nanog protein levels compared to WT mESCs when cultured in complete M10 medium (Figure 1H and EV1).

All these results support our conclusion that SIRT1 regulates the maintenance of pluripotent mESCs.

- *The Western Bolt on c-Myc-acetylation in Fig. 7G is not very convincing and should be repeated. In general, the Western Blots lack size markers. Full blots should be provided and statements on n-numbers included.*

We apologize for this technical issue. The reason why the data was not very convincing is because c-Myc runs very close to the IgG heavy chain on immuno-blots, making it difficult to detect c-Myc specific band. To obtain a better blot, we tagged the c-Myc with GFP so that it shifted significantly away from the IgG heavy chain (just like we did for N-Myc). The new blot is now shown in Figure 7G. The old blot which shows the acetylation status of endogenous c-Myc acetylation is moved to Figure EV9F.

Full blots of all immuno-blots are included as source files in the revised manuscript.

- *The in vivo experiments on embryogenesis are very interesting but somehow puzzling: Fig. 8E shows that methionine restriction aggravates developmental defects at later stages of embryogenesis, 8F shows that pre-feeding of methionine can partially rescue developmental defects thereby facilitating the birth of Sirt1-KO mice. However, this rescue does not work when initiated from E0.5 onwards but only when prefeeding is started on females before fertilization. The authors conclude that methionine supplementation rescues defects in early embryogenesis and that this is required to get life pubs of Sirt1 KOs. Does that mean that the developmental defects at later stages of embryonic development are not lethal? Are those defects implemented during early stages of development inducing lethality by inducing other developmental defects in alte embryognesis that cannot be rescued by methionine supplementation at later stages? I would assume that defects in early development would not lead to embryos that develop into later stages. The authors should explain this more carefully and if possible include a description on morphology and defects at early and late stages of embryogenesis and how these are rescued by methionine supplementation.*

This is a complicated question. We have analyzed the development of SIRT1 KO embryos at different stages, including pre-implantation embryos using in vitro culture systems. Morphologically, SIRT1 KO embryos on the pure C57BL/6J background developed normally up to E9.5 when dams were kept on the chow diet. They started to show defects in growth and eye development from E11.5, by E14.5 almost all KO embryos were significantly smaller than control littermates (Please see supplementary Figure 4 in Tang et al., 2014, Mol Cell 55: 843-55). By E18.5, they displayed delay in bone development and defects in sarcomere alignment/organization in the heart. KO pups can be born but all of them died one day after birth. So SIRT1 appears to be required at the different developmental stages for different functions. Based on the above observations as well as the pre-requirement of methionine at early stages of development, we believe that the defective methionine metabolism in SIRT1 KO embryos impacts epigenetic and redox status at the preimplantation stage, which does not result in direct morphological abnormalities at the same stage but instead affects later development and newborn survival. We have an ongoing study to investigate the developmental time point at which SIRT1

is absolutely required for mouse embryogenesis by working with an inducible SIRT1 KO mouse model. Hopefully we can have a clear idea late this year.

One thing we would like to point out is that dietary methionine supplementation did not rescue all SIRT1 KO embryos. So far, we have obtained 7 surviving pups out of 12 litters (Figure 8F and Table EV4). As shown in Figure EV10, these surviving pups still have growth retardation and eye developmental defects, just like those KO animals survived on the mixed genetic background (McBurney, MCB, 2003; Cheng et al., PNAS, 2003). We dissected 2 out of 7 surviving pups at P1.5 for immunoblotting analysis of H3K4m3 levels (as shown in Figure 8G). 2 out of the remaining 5 survived to P11.5 and P15.5, and the remianing 3 are still alive (12-week, P20.5, and P20.5 of age, respectively). We should be able to analyze their detailed morphological development once we obtain sufficient number of surviving KO animals. We are also in the process of generating transgenic mouse lines that overexpress Mat2a, which will help to directly test the importance of methionine metabolism and SAM production in SIRT1 deficiency induced development defects.

We have added a paragraph of discussion on Page 19-20 for the above points.

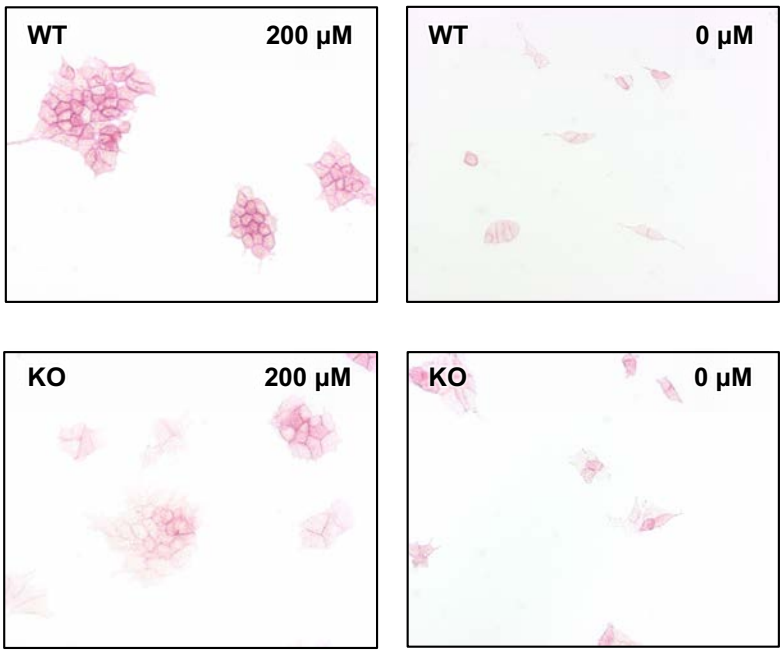

Figure R1. WT and SIRT1 KO mESCs cultured in a M10 medium containing 10% dialyzed serum. Cells were cultured for 48 hours in the medium and stained for the AP activities.

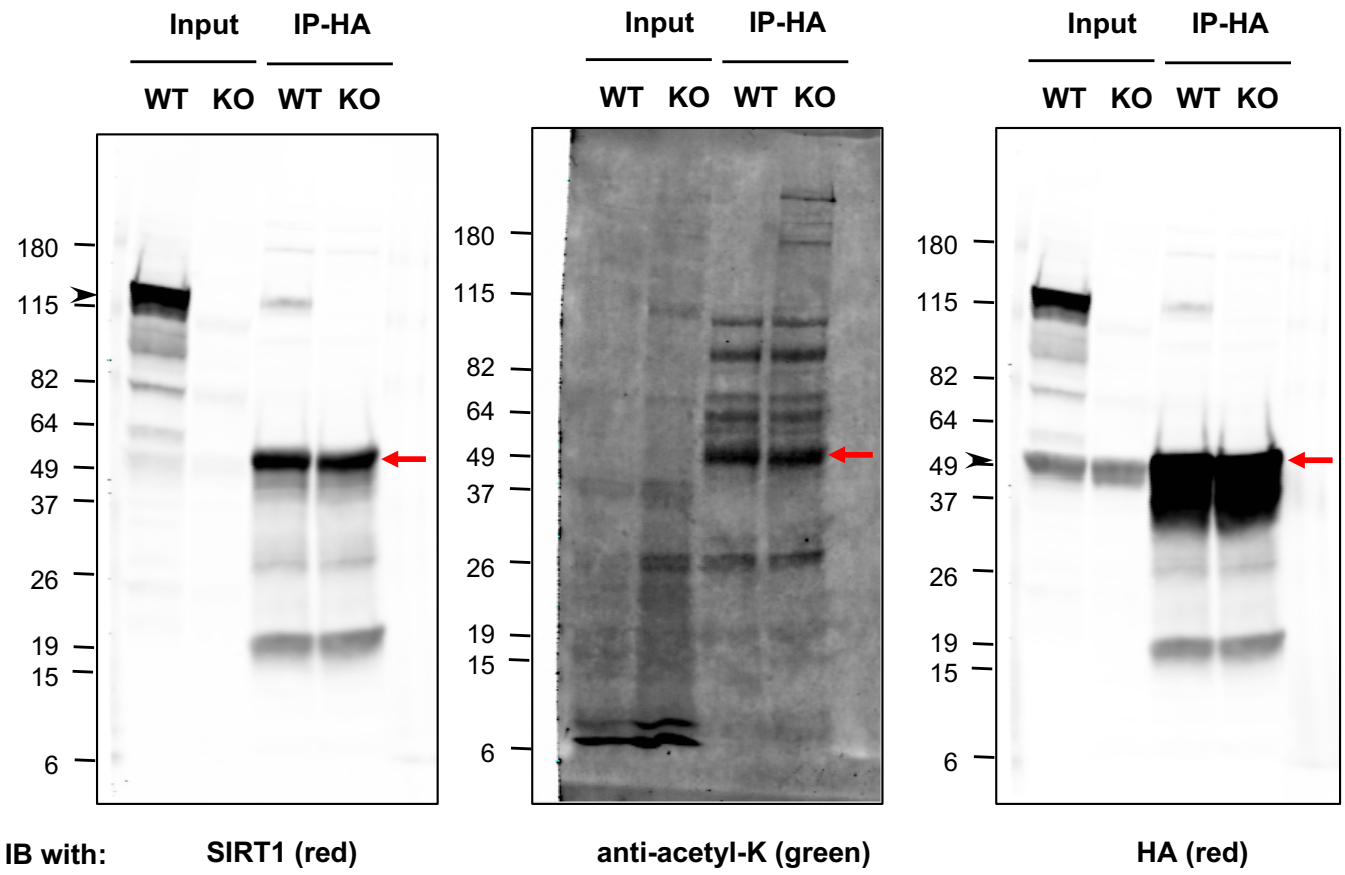

Figure R2. The interaction between SIRT1 and HA-MAT2A. WT and SIRT1 KO mESCs were iinfected with lentiviruses expressing HA-MAT2A. Total cell lysates were IP with anti-HA beads (mouse monoclonal). The membrane was probed with anti-SIRT1 antibody (mouse monoclonal, the secondary antibody was conjugated with red fluorescent dye), anti-acetyl-K antibody (rabbit polyclonal, the secondary antibody was conjugated with green fluorescent dye), and anti-HA antibody (mouse monoclonal, the secondary antibody was conjugated with red fluorescent dye) sequencially. Please note that membrane was not stripped between each blotting, so SIRT1 was visible in the anti-HA blot. Black arrow aheads, indicated SIRT1 and HA-MAT2A proteins; red arrows, IgG heavy chain.

2nd Editorial Decision 04 September 2017

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that they are in favor of publication.

REFEREE REPORTS

Referee #1:

The authors have done an excellent works to address my concerns. Now I have no more concerns and think that the manuscript is worth while to be published.

Referee #2:

The authors have carefully addressed the reviewers concerns and the paper is now suitable for publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xiaoling Li
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-96708

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Page 23
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Page 23
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, simple randomization procedure was performed to minimize bias. We randomly put the female SIRT1+/- mice under different groups according to their mice ID. Page 22
For animal studies, include a statement about randomization even if no randomization was used.	Page 23
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 23
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For cellular data, the sample size is small, so the two-tailed, unpaired, non-parametric Mann-Whitney test was used to determine the differences between means. For animal studies, the data meet the assumption of the tests. Page 29-30.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	No.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the antibodies used in this study are commercialized. We have put all the company name and catalog number in the methods part of our manuscript. We also personally validated many of key antibodies in our study by blotting them against overexpressed proteins.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Page 22-23
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 23
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Page 23

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Page 30
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Dataset was deposited. The key gene lists were also provided in Table EV2.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.