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m6A-mediated alternative splicing coupled with nonsense-mediated mRNA decay regulates SAM synthetase homeostasis

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1st Editorial Decision 2nd Oct 2020

2nd Oct 2020

Re: EMBOJ-2020-106434
Alternative splicing through m6A modification at a 3' splice site for SAM synthetase homeostasis

Dear Dr. Kuroyanagi,

Thank you for submitting your manuscript for consideration by The EMBO Journal. Please also excuse the delay in communicating the decision to you, as mentioned previously, this was due to delayed referee reports. We have now however received comments on your study from three experts, which are included below for your information.

As you will see, the reviewers are overall positive and appreciate the proposed model for the regulation of SAM synthetase by m6A-induced alternative splicing. Nonetheless they also raise several concerns, that would need to be addressed in a revised manuscript. In particular, as referee #2 and referee #4 point out, it will be important to add experiments demonstrating the role of this mechanism in a wildtype background. Referee #2 suggests a specific experiment that could be used (point 1) to monitor SAMS protein levels, but the assays referee #4 refers to should also be analyzed (Fig. 2, 3, 4). In addition, referee #3's concerns regarding the smg-2 mutant used, must be addressed, and a potential role of aberrant RNAi on the reported phenotypes excluded (ref #3-point 1). Here, the observed m6A levels and in vitro vs. in vivo data will also need to be discussed in detail (ref #3- point 2). All other concerns the referees raise, should also be carefully considered, and the manuscript revised accordingly. If you are able to resolve the key concerns and satisfactorily respond to all other issues raised by the reviewers, we will be happy to consider the study further for publication. Therefore I would now like to invite you to prepare and submit a revised manuscript.

Please note that it is our policy to allow only a single round of major revision. We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that an experimental revision may be delayed. We can extend the revision time when needed, and we have extended our 'scooping protection policy' to cover the period required for a full revision. However, it is nonetheless important to clarify any questions and concerns at this stage and we encourage you to discuss a revision plan and any potential issues you may foresee as soon as possible.

Please also feel free to contact me should you have any other further questions. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Kind regards,

Stefanie Boehm

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Editor
The EMBO Journal

Referee #2:

In this study, Watabe and colleagues perform direct sequencing of mRNA from wild type and NMD defective (smg-2) mutant animals using Nanopore technology. Analysis of these data identified novel isoforms not detected in current annotated gene models, and hundreds of target transcripts likely regulated by alternative splicing coupled with NMD (AS-NMD). Among the targets, NMD-sensitive isoforms were detected from the S-adenosyl-L-methionine (SAM) synthetase (sams) genes. Further study of the mechanism of how these isoforms are regulated revealed several interesting observations. First, the sams transcripts are differentially spliced in response to a bacterial food source, leading to the increased production of non-productive isoforms. Second, both SAM and the methyl-transferase METT-10 were found to play a key role in the homeostatic control of productive sams transcript levels, through the generation of an m6A modification on the invariant A nucleotide at a critical 3' splice site.

I found this work to be a tour-de-force study. The authors make use of a variety of approaches (nanopore sequencing, bioinformatics and functional genomics, in vitro assays, genetic mutant analysis, mass spectrometry) to uncover a very interesting and novel mechanism demonstrating the use of an important metabolite (SAM) in a negative feedback loop regulating its biosynthetic enzyme through an m6A modification of the enzyme's pre-mRNA. I believe these results will be of general interest to researchers in the gene expression community, as we are still gaining an appreciation of the consequences of the m6A modification on RNA metabolism. The current study provides insights into how this modification can impact splicing by direct modification of the 3' splice site in a physiologically relevant manner.

In general, the experiments are well conducted. One caveat to the study is that much of the downstream experiments were performed in a smg background without similar comparisons/treatments of wild type animals. I appreciate that the smg-2 mutants were used to allow the NMD-sensitive isoform to be more easily visualized, but it would be nice to know the extent to which this homeostatic control mechanism influences the levels of the SAMS proteins in a wild type animal. It would also be interesting to know more definitively if it is SAM from the E. coli food source that is triggering the large splicing pattern change. I suggest two experiments that should not be too difficult to perform to strengthen the model proposed by the authors.

1) The authors should perform a western blot to monitor SAMS1, SAMS3, and SAMS4 in the absence and presence of OP50 food in a wild type animal. Based on the model from the authors, I would expect the levels of one or more of these proteins to be reduced upon exposure to food. This should not be too difficult an experiment given that the authors have specific antibodies and conditions for western blotting. These experiments would demonstrate more definitively that this regulatory mechanism has important consequences at the level of SAMS proteins in a wild type animal.

2) It would be interesting to see if feeding SAM directly to the worms in the absence of E. coli can reproduce the effects seen with E. coli. This experiment would clearly indicate that it is very likely SAM provided by the E. coli that triggers the homeostatic regulatory effect observed in the study.

Minor points:

1) Can the authors speculate whether this mechanism is more general, or very specific to the sams genes? This may be beyond the scope of the current study, but have the authors scanned the genome for the METT-10 stem loop motif in other pre-mRNA transcripts and 3' splice sites? This type of analysis may identify other targets of METT-10-mediated m6A modification.

2) As a non-expert, I found the CID spectra panels difficult to interpret without a bit more explanation in the figure legend. For example, what do the y, c, and w labels signify? Please provide a bit more detail here.

3) In supplementary figure S8, are the panels reversed? The m6A species appears to be labeled in panel B, but in the legend this is listed as the unmodified sample. Or perhaps I am not understanding these plots?

4) The justification and description of the machine learning approaches would benefit from a few more details in the methods section. Why were these particular approaches used? Why are the results of the gradient boosting approach shown in the main figure over other machine learning methods used which appear to be performing at comparable levels?

Referee #3:

This manuscript has to potential to provide a novel resource for the global role of nonsense-mediated decay in C. elegans. In addition, the authors make claims regarding m6A which I think should be removed unless they can be substantiated. I have to major concerns that I think need to be addressed prior to publication:

1. In C. elegans there is a very unfortunate history of misinterpretation of NMD and RNAi pathways. This is due to the fact that the NMD smg-2 mutant was later found to be also mutated in the major RNAi pathway gene MUT-16. MUT-16 has a major effect on the transcriptome in general and ribosomal RNA specifically. Can the authors confirm that the smg-2 mutants analysed here are indeed wild-type for MUT-16? If this is not the case, the interpretation of their results (and their previous paper) would have to be reexamined. Hopefully this is not the case. This issue has let to a number of issues in the published literature and was described e.g. in the following paper: mut-16 and other mutator class genes modulate 22G and 26G siRNA pathways in Caenorhabditis elegans Chi Zhang, Taiowa A. Montgomery, Harrison W. Gabel, Sylvia E. J. Fischer, Carolyn M. Phillips, Noah Fahlgren, Christopher M. Sullivan, James C. Carrington, and Gary Ruvkun PNAS January 25, 2011 108 (4) 1201-1208; https://doi.org/10.1073/pnas.1018695108.

2. I am extremely concerned about the in vivo m6A data, which are very weak and seem to be in disagreement with the low levels of this modification in RNA reported by others: N6-adenosine

methylation of ribosomal RNA affects lipid oxidation and stress resistance Liberman N, O'Brown ZK, Earl AS, Boulias K, Gerashchenko MV, Wang SY, Fritsche C, Fady PE, Dong A, Gladyshev VN, Greer EL Science Advances, 2020 - Journal Article. The supplement show C. elegans mRNA has 0.0008% m6A. In comparison human cell mRNA m6A levels are around 0.2-0.4%. C. Elegans has 1000X less m6A on mRNA in comparison to humans and other organisms like flies. This is so important as the in vitro data are not linked to the in vivo data and the phenotype of metal-10 remains entirely unclear.

Referee #4:

Watabe and colleagues describe the regulation of the SAM synthetase (sams) genes in C. elegans through alternative splicing. This occurs through m6A RNA modification by the methyltransferase METT-10 at a conserved consensus sequence in the 3' splice site (SS). A similar mechanism had been described previously for the human SAM synthetase. The authors find that in C. elegans regulation of sams gene expression by alternative splicing is coupled with the nonsense mediated mRNA deacy (NMD). At low levels of SAM a distal 3'SS remains unmodified and splicing generates productive mRNA. At excess SAM concentrations METT-10 modifies the distal 3'SS which causes selection of the proximal 3'SS and production of NMD targets.
The findings of the authors are interesting and suggest a conserved mechanism for SAM synthetase regulation. However, some of their observation require to be strengthened see specific points below. Importantly the authors should include an N2 strain in their splicing analysis and determine half-live measurements to proof that mRNA degradation is affected. They have to test whether the m6A levels at the distal 3'SS in the pre-mRNA change depending on SAM activity.

Specific comments:
Figure 1: Is the overlap of the nanopore sequencing of smg2 with published results for N2, or with parallel nanopore sequencing of N2? The authors should clarify how they could compared smg2 and N2 data. 1E Do the 30 genes in smg2 depleted PTC isoforms harbors PTCs which can easily escape quality control?
Figure 2: Translation inhibitors can inhibit NMD, how does emetine treatment affect sams gene expression in fed and unfed N2 and smg2 strains? The authors should perform half-life measurements for the PTC containing isoforms in N2 versus smg2 strains to proof that degradation is really affected?
Figure 3: Can AS-NMD depends of SAM synthase activity be observed (at lower levels) in N2? 3B The tubulin signal seems overexposed. Is the expression of the sams2 pseudogene altered upon sams1 and 5 depletion in N2 and smg2?
Figure 4: What is the effect of mett-10 single mutant on sams genes splicing in N2 during feeding and fasting? AS-NMD is often used by splicing regulators as a mechanism to regulate their own abundance. Is METT-10 regulated through AS-NMD?
Figure 6: The authors identify changes in m6A at 3'SS which results in intron retention in the C.elegans sams genes similar to in the human MAT2A genes. Pendelton et al. suggest a nuclear degradation of the MAT2A transcript with retained introns. Can the authors test where in the cell the decay of the smg-2 stabilized sams transcripts occurs?
How many sams pre-mRNA molecules are methylated at the distal 3'SS of sams-3 and sams-4? The analysis of processed transcripts does not exclude whether pre-mRNAs with m6A at the distal 3'SS can give rise to productive mRNAs? How does the m6A levels in sams-3 and sams-4 relate to

SAM activity levels?
Figure 7: Can m6A modification reduce U2AF binding to the UUUUCAG motif in vitro or in vivo? Has binding of 3'SS in sams gene by U2AF been observed in CLIP data?
The expression of the different sams genes is regulated through a feedback loop involving alternative splicing in C. elegans. Is SAM activity regulated through alternative splicing in other organism?

We thank the Referees and Editors for their constructive comments on our manuscript. Revised words are indicated in red in the revised manuscript.

Summary of the revision:

We chose five of Supplementary Figures as Expanded View Figures (Fig EV1-5) and the others were renumbered as Appendix Figures accordingly. We analyzed expression of the *sams-3*, *sams-4* and *sams-5* isoforms in the wild-type strain upon feeding and emetine treatment to demonstrate that the NMD isoforms are actually expressed in the wild-type background (Appendix Fig S3). We analyzed expression of *sams-3* and *sams-4* productive mRNA isoforms and proteins in the *sams-1* single mutant (Appendix Fig S4) to demonstrate that the negative feedback regulation of the SAM synthetase also functions in the wild-type background. We demonstrate gene structure and a novel NMD isoform of the *mett-10* gene (Appendix Fig S6B-C) to demonstrate that *mett-10* is also regulated by AS-NMD. We also analyzed expression of *sams-3* and *sams-4* productive mRNA isoforms and proteins in the *mett-10* single mutant (Fig EV4) to demonstrate that METT-10 also functions in the wild-type background to repress *sams* expression. We replace modeled structure of UAF-2 binding to a 3'SS sequence with that modeled after recently published crystal structure of U2AF23 from *S. pombe* binding to the RNA (Fig 7B and Appendix Fig S11). We demonstrate nucleotide sequence alignment of intron 2-exon 3 of the *sams* genes from the genus *Caenorhabditis* (Appendix Fig S12) to speculate that the same AS-NMD regulation would function in other nematodes.

Point-by-point responses to the Referees' comments:

Referee #2:

In this study, Watabe and colleagues perform direct sequencing of mRNA from wild type and NMD defective (smg-2) mutant animals using Nanopore technology. Analysis of these data identified novel isoforms not detected in current annotated gene models, and hundreds of target transcripts likely regulated by alternative splicing coupled with NMD (AS-NMD). Among the targets, NMD-sensitive isoforms were detected from the S-adenosyl-L-methionine (SAM) synthetase (sams) genes. Further study of the mechanism of how these isoforms are regulated revealed several interesting observations. First, the sams

transcripts are differentially spliced in response to a bacterial food source, leading to the increased production of non-productive isoforms. Second, both SAM and the methyl-transferase METT-10 were found to play a key role in the homeostatic control of productive sams transcript levels, through the generation of an m6A modification on the invariant A nucleotide at a critical 3' splice site.

I found this work to be a tour-de-force study. The authors make use of a variety of approaches (nanopore sequencing, bioinformatics and functional genomics, in vitro assays, genetic mutant analysis, mass spectrometry) to uncover a very interesting and novel mechanism demonstrating the use of an important metabolite (SAM) in a negative feedback loop regulating its biosynthetic enzyme through an m6A modification of the enzyme's pre-mRNA. I believe these results will be of general interest to researchers in the gene expression community, as we are still gaining an appreciation of the consequences of the m6A modification on RNA metabolism. The current study provides insights into how this modification can impact splicing by direct modification of the 3' splice site in a physiologically relevant manner.

In general, the experiments are well conducted. One caveat to the study is that much of the downstream experiments were performed in a smg background without similar comparisons/treatments of wild type animals. I appreciate that the smg-2 mutants were used to allow the NMD-sensitive isoform to be more easily visualized, but it would be nice to know the extent to which this homeostatic control mechanism influences the levels of the SAMS proteins in a wild type animal. It would also be interesting to know more definitively if it is SAM from the E. coli food source that is triggering the large splicing pattern change. I suggest two experiments that should not be too difficult to perform to strengthen the model proposed by the authors.

We appreciate the Referee's positive and constructive comments.

1) The authors should perform a western blot to monitor SAMS1, SAMS3, and SAMS4 in the absence and presence of OP50 food in a wild type animal. Based on the model from the authors, I would expect the levels of one or more of these proteins to be reduced upon exposure to food. This should not be too difficult an experiment given that the authors have specific antibodies and conditions for western blotting. These experiments would demonstrate more definitively that this regulatory mechanism has important consequences at the level of SAMS proteins in a wild type animal.

Thank you for your constructive comments. To investigate the feedback regulation of the *sams* genes in the wild-type background, we first analyzed alternative splicing patterns of the *sams-3*, *sams-4* and *sams-5* genes in the wild-type strain N2 in the absence and presence of OP50. We also tested effect of emetine that inhibits mRNA translation and eventually represses NMD on the NMD isoforms of the *sams* mRNAs. The results of semi-quantitative RT-PCR and RT-qPCR are demonstrated in Appendix Figure S3 and described in Results as follows: "We confirmed that the PTC-containing *sams-3* and *sams-4* isoforms are also induced upon feeding and actually degraded by NMD in the wild-type background by feeding L1 larvae of N2 in the absence and presence of a translation inhibitor emetine that eventually inhibits the translation-dependent NMD process (Appendix Fig S3)." (lines 180-184).

We then analyzed effect of the *sams-1* mutation on expression of the *sams-3*, *sams-4* and *sams-5* genes in the wild-type background by RT-qPCR analysis and Western blotting. The results are demonstrated in Appendix Figure S4 and described in Results as follows: "Consistent upregulation of SAMS-3 and SAMS-

4 mRNAs and proteins in the *sams-1* mutant was observed in the wild-type background (Appendix Fig S4)." (lines 240-242). We confirmed upregulation of the mRNA and protein levels of SAMS-3 and SAMS-4 in the *sams-1* mutant upon feeding, which is consistent with the change in the alternative splicing regulation in the *smg-2; sams-5; sams-1* mutant (Fig 3).

We believe all these results support our model that SAM synthetase activity negatively regulates SAMS protein levels by modulating alternative pre-mRNA splicing of the *sams* genes.

2) It would be interesting to see if feeding SAM directly to the worms in the absence of E. coli can reproduce the effects seen with E. coli. This experiment would clearly indicate that it is very likely SAM provided by the E. coli that triggers the homeostatic regulatory effect observed in the study.

As suggested by the Referee, we treated the *smg-2* mutant with 25 mM SAM in the absence of OP50 and analyzed the splicing patterns of the *sams* genes by RT-PCR and RT-qPCR (n=3). However, there was not a remarkable effect (data not shown). This was likely because extracellular SAM does not penetrate the worm cells or because a remarkable change in the splicing patterns requires feeding-induced upregulation of the *sams* genes. We therefore could not conclude whether SAM from *E. coli* plays roles in the homeostatic regulation of the *sams* genes in *C. elegans*.

Minor points:

1) Can the authors speculate whether this mechanism is more general, or very specific to the sams genes? This may be beyond the scope of the current study, but have the authors scanned the genome for the METT-10 stem loop motif in other pre-mRNA transcripts and 3' splice sites? This type of analysis may identify other targets of METT-10-mediated m6A modification.

The NMD isoforms of the *sams* mRNAs are so far the only known target mRNAs for METT-10. A recent study demonstrated that only 0.0008% of adenines in mRNAs are m^6A-modified in *C. elegans* (Sci Adv. 6: eaaz4370, 2020), suggesting that the m^6A modification is rare, if any, in other mRNAs. To search for putative m^6A modification sites at the invariant AG nucleotide of 3'SSs involved in alternative splicing, we analyzed 61,770 unique 3'SSs detected by the Nanopore direct RNA sequencing and found 8,914 having a loop structure harboring the AG dinucleotide. Of them, 158 matched the consensus sequence TACAGA (Fig 4A) and 10 of them were relevant to alternative splicing regulation. Further direct RNA sequencing of mRNAs from the *mett-10* mutant and semi-quantitative RT-PCR analysis or the candidate genes would answer the Referee's question, but it is beyond the scope of this study and we have not done in the revision.

2) As a non-expert, I found the CID spectra panels difficult to interpret without a bit more explanation in the figure legend. For example, what do the y, c, and w labels signify? Please provide a bit more detail here.

We are sorry for any inconvenience. We added explanations to the legend for Figure 5B as follows: "(B) The negatively-charged ions of RNase T1 fragment is decomposed in the instrument by collision-induced dissociation (CID) using helium gas. The product ions produced by CID are assigned on the sequence illustrated on the top right inset panel. Nomenclature of the product ions are described in the literature

(McLuckey et al, 1992). Product ions of c and y series derive from the 5' and 3' termini of the fragment, respectively." (lines 1,098-1,103).

3) In supplementary figure S8, are the panels reversed? The m6A species appears to be labeled in panel B, but in the legend this is listed as the unmodified sample. Or perhaps I am not understanding these plots?

Thank you for the important point. Yes, the panels were reversed. We replaced the panels in Supplementary Figure S8 (renumbered as Appendix Figure S7) with correct ones.

4) The justification and description of the machine learning approaches would benefit from a few more details in the methods section. Why were these particular approaches used? Why are the results of the gradient boosting approach shown in the main figure over other machine learning methods used which appear to be performing at comparable levels?

We appreciate the suggestion. We revised Methods section as follows: "To select the best performing models, we compared classifiers xgboost version 1.0.0 (https://github.com/dmlc/xgboost) (Chen & Guestrin, 2016) and LightGBM version 2.3.2 (https://github.com/microsoft/LightGBM) (Ke et al, 2017a) and those in scikit-learn version 0.22 (https://github.com/scikit-learn/scikit-learn) (Pedregosa et al, 2011). Scikit-learn includes a variety of algorithms including Decision Tree, Random Forest, Logistic Regression, K-Nearest Neighbor, SVM, Linear Discriminant Analysis, Quadratic Discriminant Analysis, Multilayer Perceptron, Gaussian Naive Bayes and Adaptive Boosting." (lines 658-666).

Because of limited space, we picked up results with only one algorithm in the main Figure. We showed the results with "gradient boosting" in the main Figure because it provided one of the best results and we now show the other results in an Expanded View Figure (Fig EV5).

Referee #3:

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1. In C. elegans there is a very unfortunate history of misinterpretation of NMD and RNAi pathways. This is due to the fact that the NMD smg-2 mutant was later found to be also mutated in the major RNAi pathway gene MUT-16. MUT-16 has a major effect on the transcriptome in general and ribosomal RNA specifically. Can the authors confirm that the smg-2 mutants analysed here are indeed wild-type for MUT-16? If this is not the case, the interpretation of their results (and their previous paper) would have to be reexamined. Hopefully this is not the case. This issue has let to a number of issues in the published literature and was described e.g. in the following paper: mut-16 and other mutator class genes modulate 22G and 26G siRNA pathways in Caenorhabditis elegans Chi Zhang, Taiowa A. Montgomery, Harrison

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We understand the situation that the *mut-16 (mg461)* allele is present in many strains from many independent laboratories, including TR1332: *smg-2(r863) I*. Our *smg-2 (yb979)* allele was independently isolated in our own laboratory (Kuroyanagi et al., MCB, 2007) and backcrossed six times with N2 from CGC. We checked the *mut-16 (mg461)* genotype in our strains N2 and KH1668: *smg-2 (yb979) I* following a PCR-based method (PNAS 108: 1201, 2011) and confirmed that neither of them carries the *mg461* allele. We therefore believe that mRNA isoforms with PTCs are more abundant in the *smg-2* mutant due to its defects in NMD.

2. I am extremely concerned about the in vivo m6A data, which are very weak and seem to be in disagreement with the low levels of this modification in RNA reported by others: N6-adenosine methylation of ribosomal RNA affects lipid oxidation and stress resistance Liberman N, O'Brown ZK, Earl AS, Boulias K, Gerashchenko MV, Wang SY, Fritsche C, Fady PE, Dong A, Gladyshev VN, Greer EL Science Advances, 2020 - Journal Article. The supplement show C. elegans mRNA has 0.0008% m6A. In comparison human cell mRNA m6A levels are around 0.2-0.4%. C. Elegans has 1000X less m6A on mRNA in comparison to humans and other organisms like flies. This is so important as the in vitro data are not linked to the in vivo data and the phenotype of metal-10 remains entirely unclear.

We thank the Referee for raising the important reference. Indeed the m^6A modification in mRNAs are much less abundant in *C. elegans* compared to that in mammals or insects. We considered that this is consistent with the fact the *C. elegans* genome lacks orthologous genes for components of the major m^6A modification enzyme complex for mRNAs such as METTL3 and METTL14.

In human cells, another methyltransferase METTL16, whose major substrate is U6 snRNA, has been shown to methylate *MAT2A* mRNA on the 3'UTR (Pendleton et al., Cell, 2017; Shima et al., Cell Rep, 2017) and the *MAT2A* mRNA is so far the only known mRNA substrate for METTL16. The data demonstrated by Liberman et al. suggested that *C. elegans* does have m^6A modification on mRNAs (Sci Adv. 6: eaaz4370, 2020) and we demonstrate in this study that it is the NMD isoforms of the *sams* mRNAs that are m^6A-modified *in vivo*. They are so far the only mRNAs with the m^6A modification in *C. elegans*. We mention these points in Introduction as follows: "In *C. elegans*, however, orthologous genes for the m^6A writers, erasers and readers mentioned above are absent from the genome (Arribere et al, 2020; Cunningham et al, 2019) and recent studies demonstrated that only a limited fraction of mRNAs would have m^6A modification (Liberman et al, 2020; van Delft et al, 2017)." (lines 95-99) and in Discussion as follows: "*MAT2A* and *sams* mRNAs are so far the only known mRNA targets for human METTL16 and *C. elegans* METT-10, respectively. Although the m^6A modification is very rare (0.0008%) in *C. elegans* mRNAs (Liberman et al, 2020), this study revealed the critical and specific role for the m^6A modification in the mRNA metabolism." (lines 414-418).

Referee #4:

Watabe and colleagues describe the regulation of the SAM synthetase (sams) genes in C. elegans through alternative splicing. This occurs through m6A RNA modification by the methyltransferase METT-10 at a conserved consensus sequence in the 3' splice site (SS). A similar mechanism had been described previously for the human SAM synthetase. The authors find that in C. elegans regulation of sams gene expression by alternative splicing is coupled with the nonsense mediated mRNA deacy (NMD). At low levels of SAM a distal 3'SS remains unmodified and splicing generates productive mRNA. At excess SAM concentrations METT-10 modifies the distal 3'SS which causes selection of the proximal 3'SS and production of NMD targets.

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We appreciate the Referee's positive and constructive comments. Please see our responses to the specific comments below.

Specific comments:

Figure 1: Is the overlap of the nanopore sequencing of smg2 with published results for N2, or with parallel nanopore sequencing of N2? The authors should clarify how they could compared smg2 and N2 data. 1E Do the 30 genes in smg2 depleted PTC isoforms harbors PTCs which can easily escape quality control?

We previously reported that proportions of NMD isoforms to the total mRNA isoforms for ribosomal protein genes are constant throughout larval development and throughout the body (Takei et al., NAR, 2016), whereas proportions of tissue-specific mRNA isoforms apparently change during larval development (Kuroyanagi et al., PLoS Genetics, 2013). We therefore assumed that the proportions of the NMD isoforms to the total mRNA isoforms for other genes also remain constant throughout development and in nascent RNAs. Because the Nanopore direct RNA sequencing is free from length bias during library preparation, we can compare variant ratios within genes in data from multiple independent experiments. Because we needed as many reads as possible for better accuracy of NMD isoform prediction, we pooled the available N2 data at different larval stages. The Nanopore direct RNA sequencing data for N2 are derived from the published paper from other groups. We explain these points Results as follows: "For comparison, we collected and pooled as many direct RNA sequencing data in a public database as possible for mRNAs from various stages of a wild-type strain N2 (Roach et al, 2020)." (lines 123-125). "Here we assumed that the proportions of the NMD isoforms within the genes remain constant throughout development and in nascent RNAs like those of ribosomal protein genes (Takei et al, 2016) and used the pooled data for N2." (lines 140-143).

Of the 30 genes with PTC-containing yet *smg-2*-depleted mRNA isoforms, 27 genes have at least one more PTC isoforms, 19 genes have only the PTC isoforms and 14 genes are common with those having PTC-containing and *smg-2*-enriched mRNA isoforms (Supplementary Information). This is why the same GO terms are enriched in these two groups (Fig 1E). The mRNAs from such genes likely contain

upstream open reading frames (uORFs), which may not be directly involved in or differentially affect NMD. We added notes to the legend for Figure 1E as follows: "Note that 14 of the 30 genes with the *smg-2*-depleted mRNA PTC isoforms are common with those having *smg-2*-enriched PTC isoforms. This is why the same GO terms are enriched in these two groups. The mRNAs from such genes likely contain upstream open reading frames (uORFs), which may not be directly involved in or differentially affect NMD." (line 1,041-1,045).

Figure 2: Translation inhibitors can inhibit NMD, how does emetine treatment affect sams gene expression in fed and unfed N2 and smg2 strains? The authors should perform half-life measurements for the PTC containing isoforms in N2 versus smg2 strains to proof that degradation is really affected?

Thank you for raising the important point. We tested effect of emetine on alternative splicing patterns of the *sams-3*, *sams-4* and *sams-5* genes in the wild-type strain N2 in the presence of *E. coli*. The results of semi-quantitative RT-PCR and RT-qPCR are demonstrated in Appendix Figure S3. We detected the PTC-containing isoforms in the wild-type strain upon feeding. We confirmed stabilization of the PTC-containing isoforms of *sams-3* and *sams-4* upon the emetine treatment, indicating that these PTC-containing isoforms are actually degraded by NMD in the wild-type background. The results are described in Results as follows: "We confirmed that the PTC-containing *sams-3* and *sams-4* isoforms are also induced upon feeding and actually degraded by NMD in the wild-type background by feeding L1 larvae of N2 in the absence and presence of a translation inhibitor emetine that eventually inhibits the translation-dependent NMD process (Appendix Fig S3)." (lines 180-184). The effect of emetine on *sams-5* is different from that on *sams-3* and *sams-4* (Appendix Fig S3A) probably because induction of *sams-5* upon feeding also depends on protein synthesis (Appendix Fig S3B).

The amounts of mRNA isoforms at a certain time point, determined by semi-quantitative RT-PCR and RT-qPCR, are functions of their synthesis rates and degradation rates, which may vary upon feeding and during development. However, a method for determining such dynamic rates has not yet been established for *C. elegans* and we did not perform the synthesis-rate or half-life measurements. Because the *smg-2* mutant is defective in NMD, we believe that the effect of emetine on the *sams* genes in the *smg-2* mutant (demonstrated in Figure 2B and EV1) is due not to NMD inhibition but solely to protein synthesis inhibition.

Figure 3: Can AS-NMD depends of SAM synthase activity be observed (at lower levels) in N2? 3B The tubulin signal seems overexposed. Is the expression of the sams2 pseudogene altered upon sams1 and 5 depletion in N2 and smg2?

To address the Referee's question, we analyzed effect of the *sams-1* mutation on expression of *sams-3* and *sams-4* in the wild-type background by RT-qPCR. The results are demonstrated in Appendix Figure S4A. We confirmed upregulation of the *sams-3* and *sams-4* productive isoforms, indicating negative feedback regulation of alternative splicing by SAM synthetase in the wild-type background. We also performed Western blotting of SAMS-1, SAMS-3 and SAMS-4 in N2 and the *sams-1* mutant during larval development. We used CBB staining for loading control. The results are demonstrated in Appendix Figure S4B. We confirmed upregulation of the protein levels for SAMS-3 and SAMS-4 in the *sams-1* mutant during larval development, consistent with their splicing regulation (Appendix Fig S4A). These

results are described in Results section as follows: "Consistent upregulation of SAMS-3 and SAMS-4 mRNAs and proteins in the *sams-1* mutant was observed in the wild-type background (Appendix Fig S4)." (lines 240-242).

We analyzed expression levels of the *sams-2* pseudogene in N2 worms upon feeding in the presence or absence of emetine by RT-qPCR. We detected remarkable stabilization of the *sams-2* mRNA by emetine (added to Appendix Fig S3B), confirming that it is actually degraded by NMD in the wild-type background. We also analyzed the *sams-2* mRNA levels in the *smg-2* (added to Fig EV2) and *smg-2; sams-5; sams-1* mutants (data not shown) with or without feeding. The *sams-2* level is higher in the *smg-2; sams-5; sams-1* mutant and is repressed upon feeding in both strains.

Figure 4: What is the effect of mett-10 single mutant on sams genes splicing in N2 during feeding and fasting? AS-NMD is often used by splicing regulators as a mechanism to regulate their own abundance. Is METT-10 regulated through AS-NMD?

As suggested by the Referee, we analyzed effect of the *mett-10* single mutation on expression of *sams-3* and *sams-4* in the wild-type background by RT-qPCR. We found that the *sams-3* and *sams-4* productive mRNAs were induced upon feeding in the *mett-10* mutant and are more abundant compared to N2 (Fig EV4A), consistent with our model in Figure 7A. We also analyzed protein levels of SAMS-3 and SAMS-4 in the *mett-10* mutant by Western blotting and confirmed consistent upregulation throughout development (Fig EV4B). These results revealed critical role for METT-10 in the AS-NMD regulation of the *sams* genes in the wild-type background and are described in Results section as follows: "We also found that the productive isoforms of *sams-3* and *sams-4* mRNAs are upregulated in a *mett-10* single mutant (Fig EV4A) and SAMS-3 and SAMS-4 protein levels are higher in the *mett-10* mutant (Fig EV4B) compared to the wild-type strain, confirming that *mett-10* is required for negative regulation of *sams-3* and *sams-4* in the wild-type background." (lines 280-284).

On alternative splicing regulation of the *mett-10* gene. Our Nanopore sequencing data indicated that *mett-10* has a novel mRNA isoform with a PTC, although it is not significantly enriched in the *smg-2* mutant due to relatively low expression of *mett-10* (Supplemental Information). The predicted NMD isoform utilizes a cryptic 3'SS that is 8 nt upstream from the canonical 3'SS of intron 3 to cause a frame shift (Appendix Fig S6B). Direct Sanger sequencing of METT-10 cDNAs amplified form N2 and the *smg-2* mutant demonstrated stabilization the putative NMD isoform in the *smg-2* mutant (Appendix Fig S6C), indicating that *mett-10* is actually regulated by AS-NMD as speculated by the Referee. Because the *mett-10 (ok2204)* mutant lacks exon 1 through the middle of intron 3 (Appendix Fig S6B), we could not analyze the effect of the *mett-10* mutation on its own AS-NMD. The ratio of the NMD to non-NMD isoforms was not affected by the *sams-5; sams-1* mutation in the *smg-2* mutant background (data not shown). We deposited the nucleotide sequence of the novel NMD isoform as METT-10c in the DDBJ/GenBank database under an accession number LC603057. We describe the novel NMD isoform METT-10c in Discussion as follows: "*mett-10* is among the genes whose novel isoforms were discovered in this study and have a putative PTC. We found that the PTC-containing isoform of *mett-10* is stabilized in the *smg-2* mutant (Appendix Fig S6B-C), confirming that *mett-10* is also regulated by AS-NMD." (lines 431-435). Another METT-10 isoform ZK1128.2b is deposited in WormBase and RefSeq. In this isoform, a 45-nt fragment is excised from exon 2 to cause an in-frame deletion of a 15-aa portion

from the enzyme. We actually obtained a cDNA clone for ZK1128.2b yet we cannot detect its signal by direct Sanger sequencing of the METT-10 cDNAs from N2 or the *smg-2* mutant, indicating its very low abundance.

Figure 6: The authors identify changes in m6A at 3'SS which results in intron retention in the C.elegans sams genes similar to in the human MAT2A genes. Pendelton et al. suggest a nuclear degradation of the MAT2A transcript with retained introns. Can the authors test where in the cell the decay of the smg-2 stabilized sams transcripts occurs?

Whereas intron 8 is retained in the human *MAT2A* mRNA upon *METTL16* knockdown (Pendelton et al., Cell, 2017), loss of m^{6}A modification at the proximal 3'SSs of the *sams* pre-mRNAs by the *mett-10* mutation leads to switches in the splice site choice and not to intron retention in *C. elegans* (Fig 7A). Therefore, the SAMS mRNAs mature regardless of the SAM level or METT-10 function and will be exported to and translated in the cytoplasm. The NMD isoforms will then be selectively degraded by NMD in the cytoplasm. We therefore do not think that nuclear poly(A)-binding protein (PABPN1) and poly(A)-polymerases (PAPα/γ)-mediated decay (PPD) (Bresson et al., PLoS Genet, 2015) plays a role in *sams* gene expression in *C. elegans*.

How many sams pre-mRNA molecules are methylated at the distal 3'SS of sams-3 and sams-4? The analysis of processed transcripts does not exclude whether pre-mRNAs with m6A at the distal 3'SS can give rise to productive mRNAs? How does the m6A levels in sams-3 and sams-4 relate to SAM activity levels?

Because half-lives of introns are very short in general, we do not have direct evidence as to what proportions of the *sams* pre-mRNAs are m^{6}A modified on their distal/productive 3'SSs when the distal/productive 3'SSs are selected. Nevertheless, we deduce from the findings below that the distal/productive 3'SSs are selected only when they are unmodified. We demonstrated that 1) in the absence of METT-10, the distal/productive 3'SS of *sams-4* is absolutely preferred (Fig 4C, lane 4). 2) in the presence of METT-10, the proximal/unproductive 3'SSs are selected in most of the *sams* pre-mRNAs upon feeding (Fig 2B, lane 2; Fig 3A, lane 2; Fig 4C, lane 2), 3) most, if not all, of the mature, unproductive *sams-3* and *sams-4* mRNAs are m^{6}A modified on the distal/productive 3'SSs (Fig 6E and EV5A). Although the proximal/unproductive 3'SS of *sams-3* is comparably selected even in the absence of METT-10 (Fig 4C, lane 4), METT-10 more strongly forces selection of the proximal/unproductive 3'SS of *sams-3* (Fig 4C, lane 2). These findings strongly suggested that the m^{6}A modification at the distal/productive 3'SSs by METT-10 is the strict determinant of the 3'SS choice for *sams-3* and *sams-4*.

According to our model, there is a competition between splicing and m^{6}A modification at the distal/productive 3'SSs of nascent *sams* pre-mRNAs (Fig 7A). At low levels of SAM or METT-10, it will take more time for m^{6}A modification, which will allow splicing machinery to select the preferable, distal/productive 3'SSs. We added description to Discussion as follows: "regulation of *sams* gene expression is based on balanced competition between splicing and m^{6}A modification (Fig 7A)" (lines 410-411).

Figure 7: Can m6A modification reduce U2AF binding to the UUUUCAG motif in vitro or in vivo? Has binding of 3'SS in sams gene by U2AF been observed in CLIP data?

To the best of our knowledge, there is not a CLIP experiment reported for *C. elegans* U2AF. Effect of base modification on U2AF binding to 5'-UUUUCAGR-3' has not yet been analyzed *in vitro*.

The first crystal structure of U2AF small subunit binding to a 3'SS sequence UAGGU has just been solved for a budding yeast *S. pombe* (Yoshida et al., Nat Commun, 2020). They demonstrated that the interface around the -2A base was intimately surrounded by amino acid residues of U2AF23 and that no space was found for N^6-methyl modification of the -2A base to interact with U2AF23. m^6A modification at the invariant AG dinucleotide of the 3'SS dramatically reduced the affinity of U2AF23 to the RNA (Yoshida et al., Nat Commun, 2020). We therefore remodeled the structure of *C. elegans* U2AF small subunit UAF-2 binding to the same RNA (Appendix Fig S11) and found that the amino group at position 6 of adenine base is similarly embedded in a pocket formed by Zn finger domain 1 (ZnF1) of UAF-2 (Fig 7B). We revised Discussion accordingly as follows: "Crystal structure of the small subunit of U2AF binding to a 3'SS sequence has been solved only for the orthologue in fission yeast *Schizosaccharomyces pombe*, U2AF23, in complex with a part the large subunit U2AF59 (Yoshida et al, 2015; Yoshida et al, 2020). It has also been demonstrated that m^6A modification of a 3'SS sequence 5'-UUAGGU-3' at the position -2 (UUm^6AGGU) dramatically decreased affinity to the U2AF23 complex *in vitro* (Yoshida et al, 2020). To ask if m^6A modification at the AG dinucleotide would also affect 3'SS recognition by *C. elegans* UAF-2, we modelled three-dimensional structure of UAF-2 binding to 5'-UAGGU-3' after its homology to *S. pombe* U2AF23 (Appendix Fig S11). The amino group of the adenine base at position -2 that is methylated upon m^6A modification is embedded in a pocket on the surface of Zn finger domain 1 (Fig 7B) and is intimately surrounded by the identical residues as *S. pombe* U2AF23 forming the pocket (data not shown), consistent with our finding that the m^6A modification at the invariant AG dinucleotide interferes with its use as the 3'SS (Fig 7A) even in the absence of a reader protein *in C. elegans*." (lines 348-362).

The expression of the different sams genes is regulated through a feedback loop involving alternative splicing in C. elegans. Is SAM activity regulated through alternative splicing in other organism?

We searched genomes of other *Caenorhabditis* species for the *sams* genes with intron 2 and found that the nucleotide sequences of intron 2 are highly conserved for introns. The sequences between the proximal/unproductive and distal/productive 3'SSs are especially conserved. We therefore reason that expression of these *sams* genes can also be regulated by m^6A modification at the distal/productive 3'SSs. We show nucleotide sequence alignment of intron 2 and exon 3 in Appendix Figure S12 and mention in Discussion as follows: "Considering that regulation of *sams* gene expression is based on balanced competition between splicing and m^6A modification (Fig 7A), it is reasonable to suggest that highly conserved nucleotide sequences flanking the distal as well as proximal 3'SSs of intron 2 (Appendix Fig S12) play critical roles in the genus *Caenorhabditis*." (lines 410-414).

1st Revision - Editorial Decision 15th Mar 2021

Dear Dr. Kuroyanagi,

Thank you for submitting your revised manuscript, we have now received the reports from the initial referees (see comments below). I am pleased to say that they overall find that their comments have been satisfactorily addressed and now support publication. Referee #4 suggests to restructure some of the figures to include the new data. Please consider this point and revise the final manuscript as you find appropriate. In addition, I would like to ask you to also address a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving your final revision. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

Referee #2:

The authors have done a sensible job of addressing my comments and feedback. I feel that the manuscript is now suitable for publication.

Referee #4:

Watabe and colleagues provide a detailed revision of their manuscript and address most of the reviewer concerns. I therefore recommend publication of this revised manuscript.
It would be beneficial for the reader if additional performed experiments would be included in the main figures of the manuscript or the the expanded view figures. This would make it easier to highlight homoeostatic control mechanism influencing the level of SAMS proteins in the wild type animal. For example: Appendix Figure S3A can be included in Figure 2B. Appendix Figure S3B could be part of Figure EV1. Appendix Figure S4 B could be part of Figure 3.

2nd Revision - Final Decision 7th May 2021

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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

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