EMBO reports

Manuscript EMBOR-2014-39636

The cAMP phosphodiesterase Prune localizes to the mitochondrial matrix and promotes mtDNA replication by stabilizing TFAM

Fan Zhang, Yun Qi, Kiet Zhou, Guofeng Zhang, Kaari Linask and Hong Xu

Corresponding author: Hong Xu, National Institutes of Health

Review timeline:

Submission date:	22 September 2014
Editorial Decision:	10 October 2014
Revision received:	14 December 2014
Editorial Decision:	05 January 2015
Revision received:	06 January 2015
Accepted:	08 January 2015

Transaction Report:

(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.)

Editor: Esther Schnapp

1st Editorial Decision 10 October 2014

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, the referees agree that the findings are potentially interesting. However, they also raise concerns and point out that the data need to be strengthened. All referees mention that TFAM phosphorylation needs to be demonstrated, and referees 3 and 4 indicate that cAMP (and PKA) levels in the mitochondrial matrix must be shown. These are the most important concerns that must be addressed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE COMMENTS

Referee #2:

Demonstration of a mitochondrial matrix cAMP signaling that regulates mitochondrial physiology by Fan Zhang et al.

In this article the authors have identified the protein prune as a mitochondrial cAMP phosphodiesterase. They show the protein is localized to the mitochondrial matrix, functions as a phosphodiesterase and not as a pyrophosphatase and affects mitochondrial cAMP levels. They show that knockdown of prune affects mtDNA levels and suggest on the basis of various experiments that this effect is the consequence of cAMP mediated regulation of TFAM protein levels. Although this study has its merits and several interesting observations such as the cell cycle effect of the mtDNA replication effect of prune, the TFAM effect is as yet not entirely convincing, not so much for lack of trying but because of the inherent problem that mtDNA levels always correlate rather well with TFAM levels.

Comments:
Many studies have shown that mtDNA and TFAM levels appear to correlate quite well and that under various conditions moderate overexpression of TFAM can increase mtDNA levels. This leaves the question what is cause and what is consequence: are TFAM levels decreased with prune because mtDNA levels are decreased OR do mtDNA levels decrease because TFAM is more rapidly degraded by prune knockdown as the authors suggest? The experiments do not really provide a definitive answer to this question, although the combined prune and PKA knockdown does suggest that phosphorylation of Drosophila TFAM as in mammals effects TFAM stability (Fig 3B and D). The authors suggest that a likely candidate for a PKA phosphorylation site in Drosophila is T56 (Ser in humans) as at least the position (though not the residue) is conserved.

1) In Fig 3C overexpressed TFAM T56A is relatively insensitive to prune knockdown which the authors present as evidence that this variant is less sensitive to proteolytic degradation. However, they do not show the control, being overexpressed wild-type TFAM (in contrast to endogenous TFAM), while both wt as T56A TFAM overexpression partially restore mtDNA levels (Fig3B).
2) Vice versa the authors should examine a phosphomimetic T56D/E mutant to show that this mutant is more prone to degradation and possibly is less able to restore mtDNA levels with prune knockdown.
3) To more clearly demonstrate a cause-consequence what should really be shown is TFAM phosphorylation, so can the authors show that Drosophila TFAM is phosphorylated, and that with e.g. a PKA knockdown this is reduced?
4) Something might be learned from a time-course. If the primary effect of a prune knockdown is TFAM destabilization by phosphorylation, one could excpect TFAM levels to decline prior to mtDNA levels. If vice versa mtDNA levels decline before TFAM levels do, the cause for TFAM decline might very well be secondary to an mtDNA replication defect.

Minor comments:

The manuscripts contains quite a few half sentences and is too much telegram style, often lacking for example articles before nouns. This should be corrected

Pg 4: 'in metazoan.' In metazoan what?
Pg 5: 'that incubated with lacZ dsRNA' should be 'that were incubated
Pg 5: '3'UTR region', delete region
Etc etc

Referee #3:

To authors
Fan Zhang et al. find that Drosophila Prune, which is previously known to affects eye color, is a mitochondrial phosphoesterase. The authors show in sophisticated ways that Drosophila Prune is located to mitochondrial matrix, breaks down cAMP in mitochondrial matrix, and subsequently stabilizes TFAM via probably decreasing its phosphorylation by mitochondrial PKA. The stabilization of TFAM is required to maintain mitochondrial DNA replication. This article clearly shows an important role of mitochondrial cAMP signaling in maintenance of Drosophila mitochondrial genome. This work is interesting and gives a new insight into the PKA-dependent phosphorylation in the mitochondrial matrix.

Specific points
(1) The authors determined a Km of the Prune for cAMP is about 19 μM. It should be shown experimentally or by a proper article that the cAMP concentration in the matrix is indeed high enough for the phosphoesterase.
(2) The authors conclude the role of TFAM phosphorylation in its stability only by mutation experiments. The phosphorylation level should be directly measured.
(3) The authors show the decrease of TFAM in the eye of prune mutant flies. They should measure as controls TFAM in other tissues where morphological defects are not observed.

Referee #4:

The manuscript by Zhang and colleagues describes a new role of the putative fly mitochondrial phosphodiesterase, prune, in regulating TFAM levels. They propose that, by degrading cAMP in the mitochondrial matrix, prune favors TFAM stabilization. The rationale for this proposed mechanism lies on previous observations by another group, showing that TFAM phosphorylation by PKA, presumably activated by cAMP, induces TFAM degradation by the Lon protease.
Overall, the findings are novel and the implications have potential significance for the understanding of the interaction between TFAM posttranslational modifications and mtDNA replication.
The authors convincingly demonstrate the mitochondrial localization of prune and its role in the maintenance of TFAM and, secondarily, on mtDNA. Furthermore, the experiment with mutagenesis at TFAM Thr56 suggest that phosphorylation of this residue may indeed be involved, consistently with previous literature.
The major questions that need to be addressed are in regard to the mechanism of prune-mediated degradation of TFAM.
1. cAMP is not transported into the matrix of mitochondria, since no transport is known to occur across the inner membrane, unless the authors can demonstrate otherwise.
2. The cAMP reporters used are not very reliable, because even small amounts of untargeted reporters, either in the cytosol or the inter membrane space, will detect cAMP outside the matrix. This has been the problem in many studies investigating cAMP localization in the matrix. Appropriate reporters should be used with very strong matrix localization signals and low expression levels. Otherwise, the burden of proof lies on the demonstration that the reporters used here are only localized in the matrix.
3. If flies do not have soluble adenylyl cyclase, what could produce cAMP in the matrix? Perhaps, soluble adenylyl cyclase has not been adequately investigated in the fly.
4. The changes in TFAM phosphorylation state and the turnover rate of phospho-TFAM in response to prune modulation should be assessed to further show that this is the mechanism of prune action.
5. Importantly, there is no demonstration that PKA resides and operates in the matrix of fly mitochondria. This is a controversial issue in mammalian cells, which is not addressed here.
Other points:
1. If prune is a bona fide mitochondrial matrix PDE, which other cAMP-dependent processes are affected by knockdown or overexpression of prune? It is unlikely that the only role of cAMP in mitochondria is TFAM degradation.
2. The relationship with PDEs in the mitochondria of other species should be addressed, especially in reference to the mechanism of TFAM regulation proposed earlier.
3. The putative physiological regulation of cAMP in mitochondria should be discussed.

Point by point responses to referees' comments:

Referee #2

"*This leaves the question what is cause and what is consequence: are TFAM levels decreased with prune because mtDNA levels are decreased OR do mtDNA levels decrease because TFAM is more rapidly degraded by prune knockdown as the authors suggest?"*

Following referee's suggestion in comment 4), we determined the time-course of TFAM and mtDNA reduction in the *prune* RNAi cells. We found that TFAM level was reduced prior to the mtDNA decline, suggesting that the TFAM reduction is the primary defect. In addition, both overexpression of TFAM and knockdown of PKA that stabilized TFAM restored the mtDNA level in *prune* RNAi cells. These epitasis analyses also support that destabilization of TFAM by PKA acts upstream of mtDNA decline.

"*1) In Fig 3C overexpressed TFAM T56A is relatively insensitive to prune knockdown which the authors present as evidence that this variant is less sensitive to proteolytic degradation. However, they do not show the control, being overexpressed wild-type TFAM (in contrast to endogenous TFAM), while both wt as T56A TFAM overexpression partially restore mtDNA levels (Fig3B)."*

We generated the stable cell lines expressing wild type TFAM, TFAMT56A or TFAMT56D and tested their stabilities after *prune* knockdown. We found that overexpressed TFAM is still sensitive to *prune* RNAi. Both TFAMT56A and TFAMT56D are insensitive, while the protein level of TFAMT56D is significantly less than TFAM or TFAMT56A. These results are included in the revised manuscript, Figure 4, panel C, Figure S4, panel C.
We also compared mtDNA levels in these cell lines after *prune* knockdown, indeed TFAMT56D showed the least increase of mtDNA level. Overall, all three TFAM variants restored mtDNA level in *prune* RNAi cells, presumably due to the overexpression of TFAM as the referee pointed out. These results are included in the revised manuscript, Figure 3, panel B.

"2) Vice versa the authors should examine a phosphomimetic T56D/E mutant to show that this mutant is more prone to degradation and possibly is less able to restore mtDNA levels with prune knockdown."

Please refer to our response to 1).

"3) To more clearly demonstrate a cause-consequence what should really be shown is TFAM phosphorylation, so can the authors show that Drosophila TFAM is phosphorylated, and that with e.g. a PKA knockdown this is reduced?"

We knocked down *prune* or *PKA* in the cells stably expressing TFAM-V5 fusion protein. We purified TFAM-V5 using anti-V5 beads and probed with anti-V5 or anti-Phospho-(Ser/Thr) PKA substrate antibodies. While TFAM protein level was lowest in *prune* RNAi cells, it showed the highest level of PKA phosphorylation. On the other hand, PKA knockdown increased TFAM level; the TFAM phosphorylation was barely detectable. These results are included in the revised manuscript, Figure 4, panel A.

"4) Something might be learned from a time-course. If the primary effect of a prune knockdown is TFAM destabilization by phosphorylation, one could excpect TFAM levels to decline prior to mtDNA levels. If vice versa mtDNA levels decline before TFAM levels do, the cause for TFAM decline might very well be secondary to an mtDNA replication defect."

We determined the time-courses of the levels of *prune* mRNA, TFAM protein, and mtDNA after the addition of *prune* dsRNA. While the two isoforms of TFAM showed slightly different stabilities after *prune* knockdown, their levels were both decreased following the decline of *prune* mRNA level, but prior to the reduction of mtDNA level. We included these results in the revised manuscript, Figure 3, panel C and D. We also revised the text accordingly on page 9, line 2 from bottom to page 10, 1st paragraph.

"Minor comments: *The manuscripts contains quite a few half sentences and is too much telegram style, often lacking for example articles before nouns. This should be corrected"*

We appreciate the referee's effort and time to improve our manuscript. We have corrected all the grammatical errors that were pointed out by the referee. We also asked native English speakers to proofread the revised manuscript.

Referee #3

"(1) The authors determined a Km of the Prune for cAMP is about 19 μM. It should be shown experimentally or by a proper article that the cAMP concentration in the matrix is indeed high enough for the phosphoesterase."

We appreciate the referee's comment. It is indeed an important point and should be addressed. As suggested by referee #4 also, we determined that the concentration of cAMP in *Drosophila* mitochondria was 18.8 ± 3.5 pmol per mg protein. It has been reported that the water content in wet mitochondrial preparations from mammalian tissues is ~ 1 μl/mg protein (Lund P and Wiggins D, 1987, Biosci Rep 7:59-66, and the references within). Assuming that mitochondrial water content is similar between mammals and *Drosophila*, we estimate that the cAMP level in *Drosophila* mitochondria is ~ 20 μM. We included the data in the revised Figure 2, panel C, and revised the main text accordingly on page 9, line 6-10.

"(2) The authors conclude the role of TFAM phosphorylation in its stability only by mutation experiments. The phosphorylation level should be directly measured."

We determined the TFAM phosphorylation level. Please refer to our response to referee #2's comment 3).

"*(3) The authors show the decrease of TFAM in the eye of prune mutant flies. They should measure as controls TFAM in other tissues where morphological defects are not observed."*

We compared the levels of TFAM in eye and thorax that did not show any morphological defects. TFAM was reduced in the thorax of *prune* flies, but to a much lesser extent than the reduction in the eye of *prune*, and this minor reduction of TFAM does not seem to be detrimental. As of now, we are not sure why TFAM in eye is particularly sensitive to the loss of function of *prune*. We include the data in the revised figure 5, panel A.

Referee #4
"1. cAMP is not transported into the matrix of mitochondria, since no transport is known to occur across the inner membrane, unless the authors can demonstrate otherwise."

We fully agree with the referee on this issue. While we demonstrate that cAMP is present in the matrix of *Drosophila* mitochondria, the unsolved mystery is where it originates. The mammalian mitochondria inner membrane is impermeable to cAMP. Instead the matrix cAMP is produced by a mitochondrial soluble adenylyl cyclase (sAC). However, the current annotation reveals no sAC in *Drosophila* genome.
One possible explanation as proposed by the referee in comment 3: *"Perhaps, soluble adenylyl cyclase has not been adequately investigated in the fly*." Noteworthy, there are

38 isoforms of ACs encoded by 14 genes in *Drosophila* genome, it is possible that one or more isoforms might localize to mitochondria and produce cAMP locally.
We also applied a published protocol to test whether *Drosophila* mitochondria might be permeable to cAMP. We added cAMP to cells that were expressing a mitochondria cAMP reporter, MitoICUE3, and was pre-treated with digitonin. We found that CFP to YFP ratio of MitoICUE3 was increased upon the addition of cAMP. It suggests that cAMP can be transported from cytoplasm into the matrix of *Drosophila* mitochondria, though it might not be the way of producing cAMP inside mitochondria under physiological circumstance.
Nonetheless, we included the new data in Figure S5, panel B and discussed these possibilities in main text accordingly on page 12, 3rd paragraph.

"2. The cAMP reporters used are not very reliable, because even small amounts of untargeted reporters, either in the cytosol or the inter membrane space, will detect cAMP outside the matrix. This has been the problem in many studies investigating cAMP localization in the matrix. Appropriate reporters should be used with very strong matrix localization signals and low expression levels. Otherwise, the burden of proof lies on the demonstration that the reporters used here are only localized in the matrix."

The mitochondrial cAMP reporter, Mito-ICUE3 was constructed by fusing ICUE3 to SOD2 that is predominantly localized to mitochondria based on the imaging results in Figure S3E. Additionally, we tried to determine the level of MitoICUE3 that was retained in cytoplasm by western blot analysis. We could clearly detected MitoICUE3 in the mitochondrial fraction with an exposure time of 2s, however we could not detect any MitoICUE3 in the cytoplasmic fraction even with an exposure time of 20 min. Thus, the untargeted ICUE3 reporter will be less than 0.2%, if there were any. We included these results in Figure S3, panel D, the source data for Figure S3 and revised the text accordingly on Page 8, line 2 to line 5 from the bottom.
We observed that the ratio of CFP/YFP increased ~ 50% in cells expressing either ICUE3 or MitoICUE3 after stimulation. It is extremely unlikely that < 0.2% of untargeted MitoICUE3 would have any significant contribution to this 50% increase of CFP/YFP ratio.

"3. If flies do not have soluble adenylyl cyclase, what could produce cAMP in the matrix? Perhaps, soluble adenylyl cyclase has not been adequately investigated in the fly."

Please refer to our responses to comment 1.

"4. The changes in TFAM phosphorylation state and the turnover rate of phospho-TFAM in response to prune modulation should be assessed to further show that this is the mechanism of prune action."

We assayed TFAM phosphorylation in *prune*, *PKA,* and control RNAi cells. (Please refer to our response to referee #2's comment 3).

We also determined the time-course of TFAM turnover. (Please refer to our response to referee #2's comment 4).

"5. Importantly, there is no demonstration that PKA resides and operates in the matrix of fly mitochondria. This is a controversial issue in mammalian cells, which is not addressed here."

We applied the split-GFP reconstitution assay to determine whether PKA is localized to mitochondria. We co-expressed a PKA-nGFP-mCherry fusion protein with SOD2-cGFP in S2 cells. PKA was predominantly localized to cytoplasm based on the mCherry signal. We also observed that 10% of mCherry-positive cells show GFP signal that was colocalized with MitoTracker. This result clearly demonstrates that PKA is localized to mitochondria matrix.
Two ORFs were in a single cistron, with a virus self-cleaving T2A sequence in between.

All mCherry-positive cells would express both half-GFPs and therefore are expected to have reconstituted GFP. We are not sure why only 10% of mCherry-positive cells show GFP signal. Since a successful reconstitution relies on the random collision between two half-GFP molecules. The low frequency of reconstitution may reflect the low concentration of PKA inside the matrix, which reduces the chance of reconstitution. Alternatively, PKA might only be imported into a subset of cells under a certain condition.
We included these results in the revised manuscript, Figure S5, panel A. We also revised the text accordingly on page 12, 2nd paragraph.

Other points:

1. If prune is a bona fide mitochondrial matrix PDE, which other cAMP-dependent processes are affected by knockdown or overexpression of prune? It is unlikely that the only role of cAMP in mitochondria is TFAM degradation.

We agree with the referee that mitochondrial cAMP could impact a broad spectrum of mitochondrial physiology. Due to the space limitation, we briefly discussed this at the end of the main text on Page 13.

2. The relationship with PDEs in the mitochondria of other species should be addressed, especially in reference to the mechanism of TFAM regulation proposed earlier.

Although Prune belongs to a family of PDEs that are conserved among flies and mammals, human Prune does not localize to mitochondria. Another PDE, PDE2A2 localizes to mitochondria, and could be the functional counterpart of Prune in mammals. We discussed about this possibility at the end of the revised manuscript on page 13.

3. The putative physiological regulation of cAMP in mitochondria should be discussed.

A recent review article (Valsecchi F et al, 2014) nicely covers this topic. For the sake of brevity, and due to the space limitation, we referenced this article, and elected not to further elaborate on this subject.

2nd Editorial Decision 05 January 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referees that were asked to assess it, and I am happy to tell you that both support publication of the revised study by EMBO reports.

Can you please address the remaining concern of referee 4 in a final version of the manuscript?

Regarding statistics, please specify the test used to calculate p-values in the legends for figures 1, 2, 3, 4 and S1, and please explain what the circles and error bars represent and specify "n" in the legends of figures 2B and S3C.

I also would like to suggest a few minor changes to the title and abstract:

Title: cAMP signaling in the mitochondrial matrix regulates mitochondrial physiology
or (and I think this is better as it gives more information):
The cAMP phosphodiesterase Prune localizes to the mitochondrial matrix and promotes mtDNA replication by stabilizing TFAM

Abstract:
Compartmentalized cAMP signaling regulates mitochondrial dynamics, morphology, and oxidative phosphorylation. However, regulators of the mitochondrial cAMP pathway, and its broad impact on organelle function, remain to be explored. Here we report that Drosophila Prune is a cyclic nucleotide phosphodiesterase that localizes to the mitochondrial matrix. Knocking down prune in

cultured cells reduces mitochondrial transcription factor A (TFAM) and mtDNA levels. Our data suggest that Prune stabilizes TFAM and promotes mtDNA replication through down-regulation of mitochondrial cAMP signaling. In addition, our work demonstrates the prevalence of mitochondrial cAMP signaling in metazoan and its new role in mitochondrial biogenesis.

Please let me know whether you agree with these changes.

EMBO reports papers are accompanied online by A) a short summary of the findings and their significance (2 sentences), B) 2-3 bullet points highlighting key results and C) a synopsis image that must be either exactly 211x157 pixels or 550x150-400 pixels large. For the bigger image, the height is variable. Can you please send us all this information with the final version of your manuscript? You can either use a model figure or a key result for the synopsis image. Please note that not too much information can be depicted in the smaller size image, and that text must be readable at the final size of the image.

I look forward to seeing a final revised version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #2:

The authors have dealt very well with the referees comments and I am now enough convinced that the authors have uncovered a protein that modulates PKA by modulating mitochondrial cAMP, in turn regulating TFAM stability and thus mtDNA levels.

Referee #4:

The authors have performed new experiments and added to the discussion of the results. They have addressed the concerns raised.

One point to be clarified pertains to the silencing of TFAM (TFAM KD) in figure 5. It is unclear why, since the authors think that TFAM decline - dictated by its phosphorylation and Lon-mediated degradation- is the cause of mtDNA depletion, the TFAM KD flies have no eye phenotype. This result appears to be in conflict with the hypothesis. The authors must explain if the amount of KD was too small to cause mtDNA depletion to levels that result in mitochondrial dysfunction of if there is any other explanation. If the former, the TFAM KD western blot must be shown in figure 5.

2nd Revision - authors' response 06 January 2015

Thank you for the highly efficient handling of our manuscript. We are excited that both referees support publication of our work. We are now pleased to send in the final version of the manuscript with a few minor changes per your suggestions.

• We changed the title to “The cAMP phosphodiesterase Prune localizes to the mitochondrial matrix and promotes mtDNA replication by stabilizing TFAM”.
• We also incorporated your suggested changes in the abstract.
• We specified the test used to calculate p-value in the legends for Figure 1, 2, 3, 4 and S1. We also added descriptions of data points and errors bars, and specified “n” in the legends of Figures 2B and S3C.

Regarding the remaining concern of referee #4, it would not be surprising at all that the knockdown TFAM is not complete in the RNAi tissues. On the other hand, it is possible and very likely that Pn might regulate other aspects of mitochondrial physiology besides stabilizing TFAM, as referee #4 insinuated in his previous comments. Both could explain the synergy between the TFAM knockdown and a pn mutation in causing defective eye morphogenesis. To clarify, we added a sentence in the last paragraph on line 10-12, page

13. Now it reads "*It also suggests that Pn might regulate other aspects of mitochondrial physiology besides stabilizing TFAM and promoting mtDNA replication."* We used GMR-gal4 to knockdown TFAM in post-mitotic cells posterior to the furrow in the eye disc, the processors of retinal cells in the adult eye. It would be difficult to dissect pure GMR-expressing cells from whole eye to access the RNAi efficiency. Thus we do not pursue this experiment.

It's truly a pleasure working with you throughout the process. We hope the revised manuscript meets your approval, and we look forward to hearing from you soon.

3rd Editorial Decision 08 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

