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Regulation of mitochondrial pyruvate uptake by alternative pyruvate carrier complexes

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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.)

Editor: Andrea Leibfried

1st Editorial Decision 20 October 2014

Thank you for submitting your manuscript entitled 'Regulation of mitochondrial pyruvate uptake by alternative pyruvate carrier complexes' for consideration by The EMBO Journal. I have now received reports from all referees, which are enclosed below.

As you will see, the referees appreciate that your study provides the mechanistic basis for altered pyruvate transport under changing metabolic conditions in yeast. However, additional insight into the physiological relevance of the alternative carrier complexes and into the differential expression should be provided, and a few additional amendments are required before publication here. Both reports are very clear and constructive and given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees.
Please note that the EMBO Journal now also requires a complete author checklist to be submitted with all revised manuscripts.
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE COMMENTS

Referee #1:

Bender et al. investigated the mechanism of differential pyruvate import into yeast mitochondria during fermentative versus respiratory growth. While fermenting yeast cells metabolize pyruvate mostly in the cytosol and only need some mitochondrial pyruvate for amino acid synthesis, pyruvate is used for oxidative phosphorylation by respiring cells, necessitating its enhanced uptake into mitochondria. The authors demonstrate convincingly that differential expression of the pyruvate carrier homologs Mpc2 during fermentation and Mpc3 during respiration results in the presence of alternative heterodimeric pyruvate carrier complexes including the constitutively expressed homolog Mpc1. Further, the authors clarify a misconception regarding the topology of Mpc proteins, revealing that the constitutive and inducible carrier subunits differ in their topologies. Importantly, they show that the MPC complex present during respiration supports significantly higher pyruvate uptake rates and that this increased activity depends on the C-terminus of Mpc3.

This elegant and comprehensive study provides a mechanistic basis for the metabolic switch between fermentative growth and respiration, which is also highly relevant for understanding tumor biology. The authors employ a range of experimental approaches that are well suited for addressing the questions at hand, and furthermore, the work is well controlled and presented in a clear fashion. What is still lacking is a final confirmation that the alternative pyruvate carrier complexes indeed affect yeast physiology differently. With their plasmid-based expression system, the authors have the optimal tool at hand to determine whether the presence of MPCOX during fermentation or MPCFERM during respiration has detrimental physiological consequences (for instance for growth or oxygen consumption). If so, can the construct Mpc2C3 replace Mpc3 function?

1. The authors report a 300 kDa complex as well as higher molecular weight crosslinking products for the combination of Mpc1 and Mpc3 that, like the increased activity, is dependent on the C-terminus of Mpc3. Therefore it seems possible that a higher order assembly of MPCOX (or interaction with additional components) contributes to the higher transport activity of this carrier complex. It would be interesting if the authors could directly test for homodimeric interactions of individual Mpc homologs in the context of a functional carrier complex (for instance, Mpc3 dimerization in the presence of Mpc1).

2. There is a discrepancy regarding Mpc1 levels relative to those of Mpc2 or Mpc3 in Fig. 1A vs. supp. Fig. S1B that may relate to different growth conditions. Which growth conditions were employed for mitochondria isolation? In addition, Timon-Gomez et al (2013) report lower Mpc1 levels relative to Mpc2 or Mpc3. How do the authors rationalize these differences?
In general, the carbon source used in growth tests should be mentioned.

3. There appears to be a mistake in the representation of chimeric proteins in Fig. 5A. The C-terminus of Mpc2C3 should encompass residues 107-146, and accordingly that of Mpc3C2 should end with residue 129.

Referee #2:

This is a very elegant and seamless study showing that two different forms of the mitochondrial pyruvate channel subunit (MPC2 and 3) are expressed in yeast dependent on whether cells are grown under fermentative or oxidative conditions. These subunits integrate with the constitutively expressed MPC1 subunit and as a consequence, regulate the transport of pyruvate into mitochondria. Furthermore the authors show that the C-terminal region of MCP2 acts to enhance pyruvate uptake with MCP1. The results are clear and the manuscript is well written. My only concerns are relatively minor and are listed below.
1. There is a question regarding the significance of the work since this appears to be a yeast-specific event due to the ability of this fungus to alternate between anaerobic and aerobic conditions. The

authors do try to add a spin related to the Warburg effect in cancer cell lines but this does seem to be a stretch given that mammalian genomes do not contain alternatively expressed pyruvate carrier subunits.
2. The authors do not provide any information regarding how the subunits may be differentially expressed in the different growth mediums. Are there obvious promoter elements in the MPC2/3 genes that indicate how these genes are turned on and off based on growth conditions? If the genes are swapped such that they are placed under the different promoter elements, will this be deleterious for yeast growth?

1st Revision - authors' response 12 January 2015

Referee #1:

What is still lacking is a final confirmation that the alternative pyruvate carrier complexes indeed affect yeast physiology differently. With their plasmid-based expression system, the authors have the optimal tool at hand to determine whether the presence of MPCOX during fermentation or MPCFERM during respiration has detrimental physiological consequences (for instance for growth or oxygen consumption). If so, can the construct Mpc2C3 replace Mpc3 function?

We agree with the referee that physiological significance *in vivo* is an important point to address. Acting on the referee's suggestion, we have investigated the growth of yeast cells that constitutively express either MPC_{FERM} or MPC_{OX} in both glucose- and glycerol-containing media. In all cases, growth rates of MPC_{FERM}- and MPC_{OX}-expressing cells were virtually indistinguishable, indicating that growth rates are not affected by forced expression of either complex, irrespective of the carbon source available. These results are presented in a new supplementary Figure S5.
This result is not inconsistent with the model that we have presented as a conclusion of our work. Given that yeast cells can tolerate complete loss of the MPC by gene disruption even under oxidative conditions in rich medium, we consider it very likely that additional metabolic pathways can compensate for this, and that the low-activity MPC_{FERM} is sufficient to support growth. In this respect, we would like to point out that two recent papers show a similar scenario in cultured mammalian cells: after pharmacological inhibition or downregulation of the MPC, cells increase glutamate or fatty acid oxidation to compensate for the loss of pyruvate oxidation (Vacanti et al, 2014, Yang et al, 2014). On the other hand, additional regulatory mechanisms (for example, inactivation of pyruvate dehydrogenase downstream of the MPC) might divert pyruvate towards ethanol production in the presence of glucose, even if we express high-activity MPC_{OX} in cells. We have amended the discussion section in our manuscript as well as the model presented in Fig. 6 to accommodate the new data.
As proposed, we have also checked growth rates for the chimeric MPC containing the $Mpc2^{C3}$ protein. As we did not observe any differences, we have not included this finding in the revised manuscript to avoid confusion of readers.

1. The authors report a 300 kDa complex as well as higher molecular weight crosslinking products for the combination of Mpc1 and Mpc3 that, like the increased activity, is dependent on the C-terminus of Mpc3. Therefore it seems possible that a higher order assembly of MPCOX (or interaction with additional components) contributes to the higher transport activity of this carrier complex. It would be interesting if the authors could directly test for homodimeric interactions of individual Mpc homologs in the context of a functional carrier complex (for instance, Mpc3 dimerization in the presence of Mpc1).

Here again, we fully agree with the referee that the high transport activity of MPC_{OX} might be due to the formation of the 300K complex, a notion that is also supported by the ability of the chimeric $Mpc2^{C3}$ protein to form this complex as well as a high-activity transporter (Fig. 5).
As proposed, we have confirmed by co-immunoprecipitation that Mpc2 or Mpc3 homodimers can be detected in the presence of Mpc1 (see new supp. Fig. S2C). Our results clearly demonstrate, however, that homodimers are non-functional in pyruvate transport (Fig. 4). We propose that homodimers could have a 'reservoir' function, and that Mpc2 or Mpc3 subunits could be recruited by

Mpc1 from these homodimers to form a functional carrier. This could also explain the differences in protein abundance that are observed under certain conditions (see also point 2 below).
Additionally, our lab is in the process of performing a detailed proteomic study aimed at identifying physical interactors of the MPC that might be present in the 300K complex. We hope that the referee agrees with us that this detailed study is beyond the scope of the current paper.

2. There is a discrepancy regarding Mpc1 levels relative to those of Mpc2 or Mpc3 in Fig. 1A vs. supp. Fig. S1B that may relate to different growth conditions. Which growth conditions were employed for mitochondria isolation? In addition, Timon-Gomez et al (2013) report lower Mpc1 levels relative to Mpc2 or Mpc3. How do the authors rationalize these differences?
In general, the carbon source used in growth tests should be mentioned.

To clarify this matter, we have described our experiments in more detail in the revised material and methods section and we now provide full details of the carbon source used in each experiment. We have in addition also mentioned the carbon source that cells were grown on in the respective figure legends.
It is true that the abundance of Mpc2 seems to be increased when cells are grown on minimal medium (Fig. S1). Even though the reason for this is unclear, it seems that this particular subunit is induced under these conditions, possibly by the lack of some nutrient that is not present in minimal medium. We propose that the surplus Mpc2 is present in homodimers which are inactive but which may rapidly combine with Mpc1 to form functional carrier complexes. Mpc3 is most likely absent under these conditions because glucose was used as a carbon source.
In the paper from Timon-Gomez et al, MPC subunits were detected by western blot as TAP-tagged proteins. We have observed that the addition of larger tags (like TAP or GFP) change the expression levels to some extent (possibly by preventing protein degradation), and we believe that this may explain the differences between the two studies.

3. There appears to be a mistake in the representation of chimeric proteins in Fig. 5A. The C-terminus of Mpc2C3 should encompass residues 107-146, and accordingly that of Mpc3C2 should end with residue 129.

Our apologies for this oversight and we thank the referee for his vigilance. The mistake has now been corrected.

Referee #2:

1. There is a question regarding the significance of the work since this appears to be a yeast-specific event due to the ability of this fungus to alternate between anaerobic and aerobic conditions. The authors do try to add a spin related to the Warburg effect in cancer cell lines but this does seem to be a stretch given that mammalian genomes do not contain alternatively expressed pyruvate carrier subunits.

As we have indicated in the discussion section, it is true that the subunit exchange mechanism we have described in yeast is not conserved in mammals. Nevertheless, we show for the first time that a mechanism for regulating pyruvate transport has evolved that acts at the level of the MPC. We think it is of interest to speculate that while different mechanisms may have evolved in higher eukaryotes, e.g. by post-translational modification of the MPC proteins, controlling MPC activity has the potential to regulate cellular energy metabolism, and we feel that our data provide a precedent for this. In the revised manuscript, we have modified the discussion to emphasize the speculative nature of this argument.

2. The authors do not provide any information regarding how the subunits may be differentially expressed in the different growth mediums. Are there obvious promoter elements in the MPC2/3 genes that indicate how these genes are turned on and off based on growth conditions? If the genes are swapped such that they are placed under the different promoter elements, will this be deleterious for yeast growth?

Unfortunately, not much is known about promoter elements in the *MPC* genes. From the literature, it is known that *MPC3* is at least partly controlled by the Sko1 transcription factor, which is involved in adaptation to high osmolarity stress, and we have now included this information in the discussion section. This is also in line with the study of Timon-Gomez et al. (2013), which showed that Mpc3 is induced by high osmolarity. Furthermore, our study indicates that Mpc2 might be induced by nutrient deprivation in synthetic minimal medium, even though it is not known which transcription factor(s) mediate this effect.
Following the suggestion of the referee, we have fused the coding sequences of *MPC2* and *MPC3* to the ~400 bp located upstream of *MPC3*, which we assume contains the *MPC3* promoter region, and we have measured the effects on growth of these two constructs when expressed in the *mpc2*Δ*mpc3*Δ yeast strain. Since we did not observe any difference in growth rates and we think that the results presented in Supp. Fig. 5 are sufficient to clarify the effect of alternative MPC expression on cell proliferation, we did not include these data in the revised manuscript. We would like to point out that this particular concern raised by the referee has also been addressed by the response to referee #1 (unnumbered first paragraph).

2nd Editorial Decision 16 January 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are both satisfied with the revisions and therefore have no further objections towards publication in The EMBO Journal. I am thus happy to accept your manuscript in principle for publication here.

REFEREE COMMENTS

Referee #1:

The authors have carefully addressed all comments raised by the reviewers. This study on an exciting topic is now clearly suitable for publication in the EMBO Journal.

Referee #2:

I am generally satisfied with the author's response. The manuscript contains some interesting data although I do still have reservations about the overall significance of the work given the redundancies existing between these proteins when grown on different growth media and this does reduce the overall impact of the study.

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