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PML regulates PER2 nuclear localization and circadian function

Takao Miki, Zhixiang Xu, Misty Chen-Goodspeed, Mingguang Liu, Anita Van Oort-Jansen, Michael A Rea, Zhaoyang Zhao, Cheng Chi Lee, Kun-Sang Chang

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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.)

1st Editorial Decision 15 September 2011

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. While their reports are explicit, I would just highlight the most critical concerns here:

As you will see, the referees find your study potentially interesting, but all three highlighted the lack of quantitative and statistical analyses. This is a crucial point, indispensable for assessing the significance of your findings.

In addition, the referees suggest specific experiments to strengthen and clarify your message:

- non-specific effect of Per1 needs to be ruled out (ref 1 major point)
- co-localization studies including in SCN extracts should be performed (ref 2 point 1 and ref 3 point 2)
- the same cell lines should be consistently used across experiments (ref 2 point 3)
- discuss the controversial role of Per2 on activation vs. repression of Clock/Bmal1 transcription (ref 2 point 4)
- impact of PML loss on complex association should be elucidated (ref 2 point 5)
- role of NAM treatment on PML-PER2 interaction needs to be checked (ref 3 point 1)

Given the referees' positive recommendations, and providing that you are able and willing to address all referees' comments, we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal within 3 months (see below). 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.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors investigate here the role of PML in the mammalian circadian oscillator. They find that PML and PER2 co-localize and physically interact in tissue culture cells (Figure 1), in the central clock of the circadian clock, the SCN (Figure 2), PML enhances the transcription-activating potential of PER2 on a Per2 reporter gene in vitro (Figure 3), the nuclear transport and the transcriptional activity of PML depend on a specific, acetylated lysine residue (Figure 4), which represents a target site for deacetylation by SIRT1 (Figure 5), and finally, Pml1-/- mice have a shorter, less precise free-running period length (Figure 6). Altogether, the authors provide here compelling evidence for the interaction of PML with PER2, the impact of this interaction on the circadian oscillator and suggest a function of PML in the circadian clock.
Complexes were purified from mouse liver containing tagged versions of PER1 and PER2 (Duong et al., 2011). A similar study used fibroblasts (Brown et al., 2005). Though, no one has described yet an interaction of PER proteins with PML. I guess, this is a kinetic problem that the above-mentioned groups were purifying complexes during the repressive phase for the PER proteins (i.e. later than the interaction of PML with PER2). Hence, my question is about the relevance of the "activating" PER2 effect. Maximal co-localization of PER2 and PML is found at ZT12, which is before the supposed repressive function of the PER proteins. At the same token, the reporter gene used, Per2, would be active in this time frame. I would not rule out that the observed effect is quite specific for the Per2 gene and not a general mechanism. Unfortunately, the different accumulation profiles in the Pml-/- tissues are not helpful because they may be due to more indirect effects.

Major point:

To rule out a Per2-specific effect, some key experiments should also be probed for PER1 (which shows an even more dramatic phenotype in the liver). In essence, the data suggest that Pml-/- mice have a phenotype similar to Per1-/-/Per2-/- mice described by this group before. However, the behavioral phenotype of the Pml-/- mice is quite subtle. Hence, there must be some kind of compensatory mechanism.

Minor points:

Figure 1A: probably better to include as panel into Figure 4A.

Figure 1B: because it is an independent cell line, a no Zn++ control is necessary for the inducible

cell line.

Figure 5: I guess somewhere in the text Figure 5E and 5F got mixed up.

Figures 5C to 5G should be quantified. Especially the reduced co-immunoprecipitation of CLOCK and BMAL1 becomes doubtful if we take Figure 5I as the loading control (nuclear fraction).

Reporter gene assays: it is not clear, whether the transfection experiments were normalized to another reporter and whether this normalization was resistant to the inhibitor treatments.

Referee #2 (Remarks to the Author):

The work by Miki et al focuses on the regulation of Per2 subcellular localization by the promyelocytic leukemia protein PML. PML interacts with Per2 and regulates PER2 nuclear localization. In PML KO cells (superchiasmatic nucleus and MEF), Per2 is mainly cytoplasmic. Loss of PML results in reduced expression of Per1, Per2, Cry1, Bmal1 and Npas2, key regulators of cellular clock control. PML potentiates BMAL1/CLOCK mediated transcription in a Per2-dependent manner. Finally, PML loss alters the circadian period.
This is an interesting study that implicates the tumor suppressor PML in regulation of clock control. Novelty is indeed the main strength of this paper. However, this work has several weaknesses that somewhat diminish its impact.
General points
- This study proposes that PML regulates the nuclear localization of Per2. However, it does not show any quantification of results shown. In particular, the extent of colocalization is unclear. In addition, methods for studying nucleocytoplasmic shuttling should be employed.
- Related to this, most data included in this work do not show statistical analysis (see also below). This does not help the reviewer to fully appreciate the significance of data shown in the paper.
- Another main problem is that this study jumps from one cell model to another. Some experiments are conducted using U2OS, while others use MEFs or liver cells. This significantly weakens the impact of this study.
- The role of Per2 is controversial, as a number of studies showed that it represses the transcriptional role of BMAL1/CLOCK complex thus acting as part of a negative feedback loop. Instead, the authors propose that Per2 is an activator of the Clock complex. This important point needs to be extensively discussed.
- This study fails to study the impact of PML loss on Per2 association with known components of the complex. A recent study by Weitz group will be useful in selecting the relevant interactors. It would be sufficient to study its association with BMAL1, CLOCK and CRY.
Specific points
- Figure 1. The authors need to include experiments proving the specificity of the anti-Per2 antibody used for this study. Use of KO cells or Per2 knockdown cells is advised. In addition, single staining of Per2 in PML-depleted or PML-overexpressing cells should be shown in supplementary information.
- Figure 1. It is surprising that no data show PML/Per2 colocalization in MEFs. Why colocalization is shown only in U2OS cells and SCN? MEF data should be included.
- Figure 1. The authors need to determine whether PML loss of PML overexpression affects Per2 protein levels in addition to its subcellular localization.
- Figure 1. Colocalization experiments need to be quantified. This is a key point. As mentioned above, this analysis should be extended to MEFs and SCN. In this respect, U2OS cells are genomically unstable tumor cells that contain a modified PML nuclear body, called ALT-associated PML nuclear body. This structure contains several regulators of DNA damage response.
- Figure 2D is not clear as it is missing size bars. I could not understand whether it shows only two nuclei or more. Size bars should be included in all IF and IHC images.
- Figure 2. Explanation of the experiments shown in figure 2 onward (ZT0 etc) and relative referencing should be included in the main text, otherwise it is difficult to appreciate the appropriateness of the methodology involved.
- Figure 3. I am not convinced that luciferase assays should be performed in U2OS cells only. MEFs can be electroporated with Amaxa or other electroporator units, so it would be possible to repeat these experiments in a different cell system.
- Figure 3. Luc assays lack statistical analysis. The significance of these experiments cannot be

appreciated in the present form.
- Figure 3. As discussed above, Per2 has been reported to repress CLOK/BMAL1 transcriptional activity. Extensive discussion on this point is required.
- Figure 3. Per2m/m cells should be described in the results section.
- Figure 4A. Again it is unclear why PML/Per2 colocalization has not been studied.
- Figure 4B. Localization of PML mutants other than K487R should be included a supplementary information.
- Figure 4B. Statistical analysis of Luc assays is missing.
- Figure 4C-D. It is unclear why these panels are not part of Figure 2. The logical link between these and the previous panel is rather weak.
- Figure 5A, B. Statistical analysis is missing.
- Figure 5C-E. Densitometric analysis of Ac-PML/PML is required to better appreciate the effect of NAM or SIRT1 kd.
- Figure 5H. As above, IF data need quantification.
- Figure 5I. Blots showing Per2 subcellular distribution needs to be densitometrically analysed.
- Figure 5J. As discussed above, it is essential to study the extent of Per2 association with CLOCK and BMAL1 in PML-deficient cells.
- Figure 6. Assay used here should be better described in the results section (and relevant references highlighted).
- Figure 6. Importantly, the PML phenotype should be discussed in the context of Per2 phenotype (described in several papers).
- Figure 6. The in vivo phenotype should be discussed in the context of previous work reporting a CNS phenotype in PML KO mice.

Referee #3 (Remarks to the Author):

PER2 is a central clock component for circadian oscillation in mammals. Particularly, robust rhythmic expression of Per2 in the hypothalamic suprachiasmatic nucleus (SCN) is crucial to generate 24 hr rhythms in behavior. The precise entry of PER2 into the nucleus has been considered crucial for circadian transcriptional regulation of clock genes and clock-controlled genes. Miki et al. have identified tumor suppressor promyelocytic leukemia protein (PML) as a new regulator for PER2 nuclear entry, and demonstrated that its deficiency impaired to the entry of PER2 into the nucleus in the SCN, accompanying the dysregulated circadian rhythm in behavior. The authors have also provided an interesting model in which control of PML acetylation by SIRT1 regulates PER2 nuclear entry, while this model remains to be justified. These findings are very important for understanding the molecular machinery of clock oscillation, and I think it should be published. Before final, the following points should be clarified.

Comments:
(1) As mentioned by the authors, PML acetylation (i.e. NAM treatment) did not block PML entry into the nucleus. However, nuclear entry of PER2 was inhibited by NAM treatment. This sounds puzzling. Is the PML-PER2 physical interaction (shown in Fig. 1C, D) affected by NAM treatment (which, I believe, reflects PML acetylation)? What was shown is just a requirement of PML 448-555 region for in vitro PER2 pull-down assays (Fig. 1F). The possible underlying mechanism should be explicitly discussed.

(2) The abstract should be reconsidered with the above comments in mind. For instance, have all of the statements been proven? I am not convinced that the interaction of PML and PER2 was examined in the SCN extracts, while they appeared to immunocytochemically colocalize in a circadian time-specific manner. While NAM treatment appears to reduce nuclear PER2 (or promote its cytosolic fraction), no data have shown that this effect was really elicited through PML modification change. It can not be excluded that the observed NAM effect was independent of PML acetylation. For instance, NAM may affect BMAL1 acetylation (as the authors discussed), thereby simply resetting the phase (i.e. PER2 subcellular location) of cellular clock rhythm.

(3) Representative images are only available for the subcellular localization analysis of PER2 and PML in Fig. 1A, Fig. 1B, Fig. 2D, Fig. 4A, Fig. 4D, and Fig. 5H. No quantitative data are shown (these could be shown as supplementary even if space was limited).

(4) The numbers of experiments are not described for the statistical analysis in Fig. 2E, Fig. 4B, Fig. 5A, Fig. 5B, Fig.5F, and Fig. S7.

(5) Where is Fig. 3D? The authors mentioned it in p11.

(6) Where is the data for K490R mutation? The authors mentioned in p11 that a K490R PML mutation did not abolish PML/PER2 enhancement of BMAL1/CLOCK transcription of the mPer2-Luc promoter.

1st Revision - authors' response 06 December 2011

Referee #1 (Remarks to the Author):

The authors investigate here the role of PML in the mammalian circadian oscillator. They find that PML and PER2 co-localize and physically interact in tissue culture cells (Figure 1), in the central clock of the circadian clock, the SCN (Figure 2), PML enhances the transcription-activating potential of PER2 on a Per2 reporter gene in vitro (Figure 3), the nuclear transport and the transcriptional activity of PML depend on a specific, acetylated lysine residue (Figure 4), which represents a target site for deacetylation by SIRT1 (Figure 5), and finally, Pml1-/- mice have a shorter, less precise free-running period length (Figure 6). Altogether, the authors provide here compelling evidence for the interaction of PML with PER2, the impact of this interaction on the circadian oscillator and suggest a function of PML in the circadian clock.

Complexes were purified from mouse liver containing tagged versions of PER1 and PER2 (Duong et al., 2011). A similar study used fibroblasts (Brown et al., 2005). Though, no one has described yet an interaction of PER proteins with PML. I guess, this is a kinetic problem that the above-mentioned groups were purifying complexes during the repressive phase for the PER proteins (i.e. later than the interaction of PML with PER2). Hence, my question is about the relevance of the "activating" PER2 effect. Maximal co-localization of PER2 and PML is found at ZT12, which is before the supposed repressive function of the PER proteins. At the same token, the reporter gene used, Per2, would be active in this time frame. I would not rule out that the observed effect is quite specific for the Per2 gene and not a general mechanism. Unfortunately, the different accumulation profiles in the Pml-/- tissues are not helpful because they may be due to more indirect effects.

Major point:

To rule out a Per2-specific effect, some key experiments should also be probed for PER1 (which shows an even more dramatic phenotype in the liver). In essence, the data suggest that Pml-/- mice have a phenotype similar to Per1-/-/Per2-/- mice described by this group before. However, the behavioral phenotype of the Pml-/- mice is quite subtle. Hence, there must be some kind of compensatory mechanism.

We reasoned the referee must have meant *Per1*$^{-/-}$ rather than *Per1*$^{-/-}$/*Per2*$^{-/-}$ with respect to locomotor behavioral similarity to *Pml*$^{-/-}$ mice since *Per1*$^{-/-}$/*Per2*$^{-/-}$ mice have no circadian rhythm when released into a free-running environment (Zheng et al., 2001). Additional experiments have been performed to address the referees concern by examining phase shift responses to a light pulse using the *Pml*$^{-/-}$ mice. Studies using a protocol where a light pulse was given on the last day of an LD cycle entrainment followed by release into free-running conditions have shown that *Per1*$^{-/-}$ mice have no phase advance response, while the PER2 deficient mice have enhanced phase advance response to a ZT22 light pulse compared to wild type mice (Albretch et al., 2001; Spoelstra et al., 2004). These studies further showed that the phase delay response to a ZT14 light pulse of *Per1*$^{-/-}$ was normal while PER2 deficient mice have weakened or no phase delay response. Using a similar protocol, our studies showed that *Pml*$^{-/-}$ mice have significantly increased phase advance response to a ZT22 light pulse and a significantly weakened phase delay response to a ZT14 light pulse compared to wild type mice (See new 6F, 6G, 6H and 6I). Thus, the phase shift response of *Pml*$^{-/-}$ mice to a light pulse is more similar to that of PER2 deficient mice. The text is revised accordingly.

Minor points:

Figure 1A: probably better to include as panel into Figure 4A.

We have left this panel in its original place.

Figure 1B: because it is an independent cell line, a no Zn++ control is necessary for the inducible cell line.

A no Zn^{++} treatment control panel is now included in the revised Figure 1B.

Figure 5: I guess somewhere in the text Figure 5E and 5F got mixed up.

We have corrected this mix up in the revised manuscript.

Figures 5C to 5G should be quantified. Especially the reduced co-immunoprecipitation of CLOCK and BMAL1 becomes doubtful if we take Figure 5I as the loading control (nuclear fraction).

Quantification and statistical analysis of the results for Figures 5C to 5G are shown as supplementary Figures S15. Proteins fractions isolated from nuclear and cytoplasmic fractions were used in Figure 5I while total cell lysates were used in previous Figure 5J. Therefore, results shown in Figure 5I cannot be used as loading control for previous Figure 5J. We performed the experiment suggested by the referee and the results which show the effects of NAM on PML/PER2 interaction in wild type MEF cells is now included as the new Figure 5J. This study shows that PML and PER2 co-immunoprecipitation was significantly reduced after treatment with NAM, consistent with the immnunofluorescense analysis shown in Figure 5H (new Figure 5J). Also, new Figure 5J shows the total amount of PER2 in total cell lysates did not change significantly with or without NAM treatment.

Reporter gene assays: it is not clear, whether the transfection experiments were normalized to another reporter and whether this normalization was resistant to the inhibitor treatments.

The reporter assay was normalized to Renilla (TK-luc). A statement is now included in the Methods.

Referee #2 (Remarks to the Author):

The work by Miki et al focuses on the regulation of Per2 subcellular localization by the promyelocytic leukemia protein PML. PML interacts with Per2 and regulates PER2 nuclear localization. In PML KO cells (superchiasmatic nucleus and MEF), Per2 is mainly cytoplasmic. Loss of PML results in reduced expression of Per1, Per2, Cry1, Bmal1 and Npas2, key regulators of cellular clock control. PML potentiates BMAL1/CLOCK mediated transcription in a Per2-dependent manner. Finally, PML loss alters the circadian period.

This is an interesting study that implicates the tumor suppressor PML in regulation of clock control. Novelty is indeed the main strength of this paper. However, this work has several weaknesses that somewhat diminish its impact.

General points

- This study proposes that PML regulates the nuclear localization of Per2. However, it does not show any quantification of results shown. In particular, the extent of colocalization is unclear. In addition, methods for studying nucleocytoplasmic shuttling should be employed.

Quantifications for all relevant figures are now included. (See responses to Specific Points below).

- Related to this, most data included in this work do not show statistical analysis (see also below). This does not help the reviewer to fully appreciate the significance of data shown in the paper.

Statistical analysis of all relevant figures have been done and are now included. (See responses to Specific Points below).

- Another main problem is that this study jumps from one cell model to another. Some experiments are conducted using U2OS, while others use MEFs or liver cells. This significantly weakens the impact of this study.

The referee raised a good point about using MEF and U2OS cells in the same set of experiments given that the genomic makeup of tumor cell is very different from MEF cells. Therefore, we have

repeated the relevant reporter assay studies in wild type MEF cells (results shown in new Figure 3A, new Figure 4B and Figure S14). See detailed response to specific points below.

- The role of Per2 is controversial, as a number of studies showed that it represses the transcriptional role of BMAL1/CLOCK complex thus acting as part of a negative feedback loop. Instead, the authors propose that Per2 is an activator of the Clock complex. This important point needs to be extensively discussed.

See detailed response to specific points below.

This study fails to study the impact of PML loss on Per2 association with known components of the complex. A recent study by Weitz group will be useful in selecting the relevant interactors. It would be sufficient to study its association with BMAL1, CLOCK and CRY.

Additional experiments have been performed in response to the referee's concern. The results of these studies are now included in new Figure 5K. See detailed response to specific points below.

Specific points

- Figure 1. The authors need to include experiments proving the specificity of the anti-Per2 antibody used for this study. Use of KO cells or Per2 knockdown cells is advised. In addition, single staining of Per2 in PML-depleted or PML-overexpressing cells should be shown in supplementary information.

Characterization of the anti-PER2 antibodies has been described in our previous report (Zheng et al., 2001). In response to the referee's concern, we performed immunofluorescence staining in $Per2^{-/-}$ MEFs and a Western blot analysis using this anti-PER2 antibody (supplementary Figure S18). Negative immunofluorescent signal was found in the $Per2^{-/-}$ MEFs, and the anti-PER2 antibodies recognized a specific 170Kd protein in wild type that is absence in $Per2^{-/-}$ MEF lysates.

Immunofluorescence staining of the endogenous PER2 in $Pml^{-/-}$ and wild type MEFs is shown in Figure 1A (top panel) and supplementary Figure S1A, as well as the immunocytochemistry staining shown in Figure 2B.

Figure 4A shows co-localization of over expressed PML and PER2 in $Pml^{-/-}$ MEF. The results are consistent with Figure 1B and supplementary Figure S1B when PML was over expressed driven by IFg or by an over expressing construct driven by a Zn++ inducible promoter.

- Figure 1. It is surprising that no data show PML/Per2 colocalization in MEFs. Why colocalization is shown only in U2OS cells and SCN? MEF data should be included.

Please see Figure 4A (middle panel) for co-localization of PML and PER2 when expressed in $Pml^{-/-}$ MEF cells.

- Figure 1. The authors need to determine whether PML loss of PML overexpression affects Per2 protein levels in addition to its subcellular localization.

We reasoned that the referee must have meant "PML loss or PML overexpression" in the statement above.

Our studies showed that the loss of PML significantly reduced the expression of *Per2* and the distribution of PER2 became mainly cytosolic and perinuclear (Figure 1A, 4A, 4C, 4D, S1A,).

In contrast, over expression of PML resulted in enhanced co-localization of PML and PER2 (Figure 1B and Figures 4A, S1B)

- Figure 1. Colocalization experiments need to be quantified. This is a key point. As mentioned above, this analysis should be extended to MEFs and SCN. In this respect, U2OS cells are genomically unstable tumor cells that contain a modified PML nuclear body, called ALT-associated PML nuclear body. This structure contains several regulators of DNA damage response.

The results of quantification of Figure 1A are now shown in Figures S1A and S1B.

To address the referee's concern about PML-PER2 interaction in the SCN, we performed a co-immunoprecipitation study using lysates prepared from SCN dissected from 20 mouse brains sacrificed at ZT14. Western blot analysis showed that the anti-PER2 antibody, but not control IgG, immunoprecipitated PER2 and PML (see new Figure 2F). These new data support the results obtained from immunofluorescence staining that PER2 and PML are associated at the endogenous levels in the SCN (Figures 2D, 2E).

- Figure 2D is not clear as it is missing size bars. I could not understand whether it shows only two nuclei or more. Size bars should be included in all IF and IHC images.

Size bars are now included in all relevant figures.

Figure 2. Explanation of the experiments shown in figure 2 onward (ZT0 etc) and relative referencing should be included in the main text, otherwise it is difficult to appreciate the appropriateness of the methodology involved.

Zeitgeber Time 0 (ZT0) is when light is turned on and ZT12 is when light is turned off during a 24 h diurnal cycle. A statement is included in the Methods to clarify this point.

- Figure 3. I am not convinced that luciferase assays should be performed in U2OS cells only. MEFs can be electroporated with Amaxa or other electroporator units, so it would be possible to repeat these experiments in a different cell system.

To address the referee's concern, we have repeated the reporter assay using wild type MEFs and the results are shown in the new Figure 3A. The previous Figure 3A using U2OS cell is now supplementary Figure S7. Changes in cell type do not affect the conclusion that PML enhanced BMAL1/CLOCK transcription in the presence of PER2.

In addition, Figure 4B has also been repeated using wild type MEFs. The previous results obtained from U2OS cells are now shown in supplementary Figure S11. Again, the change in cell type did not affect the original conclusion. Figure S14 shows a repeat of the experiment in Figure 4A using wild type MEF cells. The change in cell type did not affect the original conclusion.

- Figure 3. Luc assays lack statistical analysis. The significance of these experiments cannot be appreciated in the present form.

Statistical analyses are now included in the revised manuscript.

- Figure 3. As discussed above, Per2 has been reported to repress CLOK/BMAL1 transcriptional activity. Extensive discussion on this point is required.

Extensive discussion has been included in the Discussion section. In an early report, modest inhibition by PER of CLOCK/BMAL1 driven transcription of Per1 and AVP promoter driven transcription when compared to CRY was observed (Kume et al., 1999). This study further showed that when the AVP promoter was used, the PER protein failed to inhibit BMAL1/MOP4 mediated transcription even at very high levels of PER expression driven by vector transfection. In contrast, CRY inhibition of BMAL1/MOP4 mediated transcription was equally potent as that observed for BMAL1/CLOCK transcription. Given that we now recognize that MOP4 (also known as NPAS2) is the redundant homolog of CLOCK, the conclusion from the Kume *et al.* that PER acts in the negative loop is premature.

In addition, newly published observations from Dr. A. Sancar's group also raised questions about the clock mechanism model that proposes PER2 and CRY together negatively regulate BMAL1/CLOCK:E-box mediated transcription (Ye et al., 2011). Dr Sancar's group demonstrated by reconstitution experiments with full-length recombinant proteins that PER2 blocks CRY from interacting with BMAL1/CLOCK when the heterodimer was bound to E-box DNA. The studies further showed that CRY bound to the BMAL1/CLOCK:Ebox complex was removed by the addition of PER2. These studies suggest that PER2 must act opposite to CRY in the clock mechanism

- Figure 3. Per2m/m cells should be described in the results section.

Per2$^{m/m}$ has an in-frame deletion of the PASB domain that generates a non-functional truncated PER2 (Zheng et al., 1999). A statement is included in the text with regard to *Per2*$^{m/m}$ cells

- Figure 4A. Again it is unclear why PML/Per2 colocalization has not been studied.

Figure 4A shows the cellular localization of PER2 in *Pml*$^{-/-}$ MEF under the following transfection conditions: transfected with an expression construct for *Per2* (top panel), transfected with expression constructs for wild type *Pml* and *Per2* (middle panel) and transfected with expression constructs for *K487R-PML* and *Per2* (bottom panel). Statistical analysis is now included in Figure S9.

- Figure 4B. Localization of PML mutants other than K487R should be included a supplementary information.

As requested, the localization of K460R and K515R PML in *Pml*$^{-/-}$ MEF is shown in supplementary Figure S12

- *Figure 4B. Statistical analysis of Luc assays is missing.*

Statistical analysis is now included.

- *Figure 4C-D. It is unclear why these panels are not part of Figure 2. The logical link between these and the previous panel is rather weak.*

We believe the current arrangement of data (Figures 4A, C and D) shows the importance of PML for PER2 localization into the nucleus.

- *Figure 5A, B. Statistical analysis is missing.*

Statistical analysis is now included

- *Figure 5C-E. Densitometric analysis of Ac-PML/PML is required to better appreciate the effect of NAM or SIRT1 kd.*

Densitometric analysis is now included (supplementary Figure S15).

- *Figure 5H. As above, IF data need quantification.*

Quantification is shown in supplementary Figure S17.

- *Figure 5I. Blots showing Per2 subcellular distribution needs to be densitometrically analysed*

Densitometric analysis is now included in the figure.

- *Figure 5J. As discussed above, it is essential to study the extent of Per2 association with CLOCK and BMAL1 in PML-deficient cells.*

Our new results presented in Figure 5K show that both CLOCK and BMAL1 interactions with PER2 were dampened significantly in *Pml*$^{-/-}$ MEF cells (See new Figure 5K). Thus PER2 association with CLOCK and BMAL1 is significantly reduced in PML deficient cells.

- *Figure 6. Assay used here should be better described in the results section (and relevant references highlighted).*

A brief description of the wheel running assay has been described in the Methods and the appropriate reference has been cited

- *Figure 6. Importantly, the PML phenotype should be discussed in the context of Per2 phenotype (described in several papers)*

Both the *Per2*$^{m/m}$ and *Per2*$^{-/-}$ mice produced in our laboratory have a transient period of 22 h, followed by a loss of circadian rhythm (Zheng et al. 1999; Zheng et al., 2001). Another strain of *Per2*$^{-/-}$ mice generated independently also displayed a similar phenotype (Bae et al., 2001). Thus, the circadian rhythm behavior of PML and PER2 deficient mice is different and this could be explained by some level of PER2 getting into the nucleus in *Pml*$^{-/-}$ cells as observed in Figures 1A, S1A, 4C, 4D. However, both *Pml*$^{-/-}$ and *Per2* deficient mice displayed quite similar defects in their phase shift response to a light pulse following entrainment (see response to referee #1: point #1). The text has been revised to take these issues into consideration.

- *Figure 6. The in vivo phenotype should be discussed in the context of previous work reporting a CNS phenotype in PML KO mice.*

We believe it would be premature to correlate the current findings with previous observations of PML's role in the CNS.

Referee #3 (Remarks to the Author):

PER2 is a central clock component for circadian oscillation in mammals. Particularly, robust rhythmic expression of Per2 in the hypothalamic suprachiasmatic nucleus (SCN) is crucial to generate 24 hr rhythms in behavior. The precise entry of PER2 into the nucleus has been considered crucial for circadian transcriptional regulation of clock genes and clock-controlled genes. Miki et al. have identified tumor suppressor promyelocytic leukemia protein (PML) as a new regulator for PER2 nuclear entry, and demonstrated that its deficiency impaired to the entry of PER2 into the

nucleus in the SCN, accompanying the dysregulated circadian rhythm in behavior. The authors have also provided an interesting model in which control of PML acetylation by SIRT1 regulates PER2 nuclear entry, while this model remains to be justified. These findings are very important for understanding the molecular machinery of clock oscillation, and I think it should be published. Before final, the following pointsshould be clarified.

Comments:

(1) As mentioned by the authors, PML acetylation (i.e. NAM treatment) did not block PML entry into the nucleus. However, nuclear entry of PER2 was inhibited by NAM treatment. This sounds puzzling. Is the PML-PER2 physical interaction (shown in Fig. 1C, D) affected by NAM treatment (which, I believe, reflects PML acetylation)? What was shown is just a requirement of PML 448-555 region for in vitro PER2 pull-down assays (Fig. 1F). The possible underlying mechanism should be explicitly discussed.

The loss of lysine 487 of PML prevented both the mutant PML and PER2 from entering the nucleus (Figure 4A). This demonstrates that lysine 487 is essential for PML entry into the nucleus. Although, K487R PML and PER2 were primarily cytosolic they remained largely co-localized (Figure 4A bottom panel). Thus, the loss of lysine 487 did not disrupt PML/PER2 interaction. The deletion mapping studies indicate that the carboxyl region from 448-555 of PML is important for PML/PER2 interaction (Figure 1F). NAM treatment increased acetylated-PML (Fig 5C, 5E, S16) but the acetyl-PML localized mainly in the nucleus (Figure 5H). However, PER2 nuclear distribution was significantly reduced by NAM and consequently its interaction with acetyl-PML was also reduced (Figure 5H and new Figure 5J). Therefore, from those studies we concluded that lysine 487 is critical for PML nuclear entry but its acetylation does not prevent PML from nuclear entry. The region of PML that interacts with PER2 was mapped to the carboxyl region that includes lysine 487. We surmise that the deacetylation of K487PML initiates the efficient nuclear transport of PER2. We have revised the Discussion to clarify these points.

(2) The abstract should be reconsidered with the above comments in mind. For instance, have all of the statements been proven? I am not convinced that the interaction of PML and PER2 was examined in the SCN extracts, while they appeared to immunocytochemically colocalize in a circadian time-specific manner. While NAM treatment appears to reduce nuclear PER2 (or promote its cytosolic fraction), no data have shown that this effect was really elicited through PML modification change. It can not be excluded that the observed NAM effect was independent of PML acetylation. For instance, NAM may affect BMAL1 acetylation (as the authors discussed), thereby simply resetting the phase (i.e. PER2 subcellular location) of cellular clock rhythm.

To address the referee's concern, new experiment designed to demonstrate PML-PER2 interaction in the SCN are now included. We performed co-immunoprecipitation using SCN protein extracts prepared from 20 mouse brains. As shown in the new Figure 2F, the results of this study demonstrated that immunoprecipitation with anti-PER2 antibody but not the control IgG co-immunoprecipitated PML from the SCN extract. This result is supportive of the immunocytochemical analysis which showed PML-PER2 co-localization in the SCN (Figures 2D and 2E).

Results presented in Figure 4D show that, in the absence of PML function, PER2 localized to the cytosol/perinuclear region regardless of the phase of the SCN clock. We showed that NAM increased acetylation of PML in MEF cells (see new Figure S16) but did not block its nuclear entry (Figure 5H). However, PER2 nuclear entry is significantly reduced by NAM and this is supported by reduced co-immunoprecipitation of PML-PER2 (Figure 5H, new Figure 5J). Our data cannot rule out the possibility that the phase of the clock of individual MEFs could have been reset by NAM. However, our reporter assays showed that NAM treatment did not alter the basal BMAL1-CLOCK mediated transcription of the *Per2* promoter (Figure 5A, see wild type MEF data: new Figure S14).

(3) Representative images are only available for the subcellular localization analysis of PER2 and PML in Fig. 1A, Fig. 1B, Fig. 2D, Fig. 4A, Fig. 4D, and Fig. 5H. No quantitative data are shown (these could be shown as supplementary even if space was limited).

Quantification of the respective images is included in the revised figures or as supplementary figures (see Figures S1A, S1B, S9, S13, S17). Note Figure 2E is a quantification for Figure 2D.

(4) The numbers of experiments are not described for the statistical analysis in Fig. 2E, Fig. 4B, Fig. 5A, Fig. 5B, Fig.5F, and Fig. S7.

The number of experiments used for statistical analysis is now included in the legends for all the figures mentioned.

(5) Where is Fig. 3D? The authors mentioned it in p11.

The error has been corrected.

(6) Where is the data for K490R mutation? The authors mentioned in p11 that a K490R PML mutation did not abolish PML/PER2 enhancement of BMAL1/CLOCK transcription of the mPer2-Luc promoter.

The results of this study has been included as supplementary Figure S10

We believe that we have addressed all the specific points raised by the referees. We thank the referees for their comments and suggestions that have resulted in new experiments that strengthen our findings.

Cited Publications:

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